Conservation studies with Nepenthes macfarlanei Hemsl. in peninsular Malaysia

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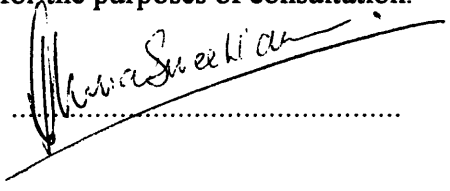
Award date:
1995

Awarding institution:
University of Bath

CONSERVATION STUDIES WITH *Nepenthes macfarlanei* Hemsl. IN PENINSULAR MALAYSIA

submitted by Lillian Chua Swee Lian
for the degree of PhD
of the University of Bath
1995



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Dedicated to

my parents

ACKNOWLEDGMENTS

I wish to thank the Board and the Director-General of the Forest Research Institute Malaysia for granting the permission to undertake this study. I would like to convey my deepest appreciation to my supervisor, Professor G.G. Henshaw and to my local supervisors, Professor H.F. Chin and Dr. H.T. Chan for the intellectual advice and guidance provided throughout the period of study. I also wish to thank the Directors of the Forestry Departments of Pahang, Perak, Johore and Trengganu for granting the permission to undertake field studies in the selected Forest Reserves.

Warmest thanks go to Dr. Ruth Kiew, Dr. Tony Lamb, Anthea Phillips and Dr. Anna Dourado for the stimulating discussion and criticism provided at the initial stages of this study. Special thanks go to Dr. L.G. Saw and Professor E. Soepadmo for their valuable advice, encouragement and enthusiasm throughout my academic endeavors.

In particular, I am indebted to Damanhuri Sabari, Angan Atan, Apok Kassim, Y.K. Fan and Baya Busu for their untiring assistance and dedication throughout the period of study.

My sincere thanks to E.S. Quah and his team from the Ecology unit for the field assistance; Dr. Baskaran Krishnapillay, Dr. Marzalina Mansor and the staff of the Seed Technology Laboratory for the support, motivation and the use of facilities; Y.F. Ho for the much-needed assistance in SEM work; Richard Chung for his contributions to the embryological work; the staff of Botany unit for the technical assistance; Dr. S.G. Khoo for the indentification of insects; Roger Guevara for the helpful ideas, constructive discussion and companionship; Chris Davey for the technical assistance and Dr. Paul Christie for the assistance in the statistical aspects.

I would like to acknowledge, with thanks, the assistance given by the management and staff of the Herbarium and the Economic and Conservation Unit of the Royal Botanical Gardens, Kew, in particular Dr. Martin Cheek and Rosemary Simpson and to Dr. Matthew Jebb of Trinity College, Dublin.

Last but not least, my warmest gratitude to Dr. Farah D. Ghani, Dr. Yurawaj Kumar, Miawia, Nazan and Suad whose rapport has provided me with much inspiration and

motivation throughout the period of study. My heartfelt thanks goes to my family whose understanding and forbearance saw the fruition of this study.

SUMMARY

The level of endemicity is an important criterion used to consider whether a particular species merits conservation attention. Although *Nepenthes macfarlanei* Hemsl. is endemic to Peninsular Malaysia, nothing is known about the level of its endemicity. This study aimed to document its present status and to recommend *in situ* and *ex situ* conservation measures.

Nepenthes macfarlanei is a widespread endemic and is distributed on certain mountain peaks in massifs located at the central and western parts of Peninsular Malaysia. Although disjunct in distribution, it is locally abundant in its range. There is a high density of seedlings and juvenile plants in the population and they establish close to the parent plants. The species has a strong affinity for ridges and plateaus, both of which are microhabitats which experience environmental extremes in terms of microclimate.

N. macfarlanei has an allogamous breeding system and is entomophilous. The potential pollinators are generalist insects, including flies, wasps, small bees and plant hoppers. Although the overall reproductive efficiency of the sample population was low, the population was capable of producing large number of viable seeds.

Seeds of *N. macfarlanei* apparently declined in viability during storage. Although fresh seeds gave a high germination percentage, the germination percentage and time declined after storage at room and at low temperatures.

N. macfarlanei may be easily propagated through the *in vitro* method. Cotyledonary seedlings and half-strength Murashige & Skoog, supplemented with 5×10^{-5} M BAP were the most suitable explants and medium respectively. The most suitable auxin for the rooting of shoot buds was NAA.

In terms of its present conservation status, *N. macfarlanei* perhaps should not be considered vulnerable. This status may however, deteriorate if attempts are not made to reduce large-scale forest fragmentation. Although populations were observed to be thriving in at least one fragmented habitat, certain features of this species indicate that its populations would become extinct if more massive fragmentation occurs. The propensity

of the seedlings to establish themselves close to parent plants and the affinity to certain microhabitats indicate the lack of colonization capacity and that founder events are not likely to happen when the population becomes extinct. The species' dependence on insects for pollination and the observed low frequency of insect visitations indicate that habitat changes could influence the reproductive and regeneration capacities of the species through their negative impacts on insect populations. At the present time, *ex situ* measures such as long-term germplasm storage cannot be recommended for *N. macfarlanei*. It is therefore recommended that *in situ* conservation, through the establishment of a network of protected areas be pursued by the relevant authorities. By extrapolation, it can be assumed that this approach would be particularly crucial for the hyper-endemic *Nepenthes* species in Sabah and Sarawak. In cases where the loss of the population is imminent, rescue and recovery work may be undertaken with simple micropropagation techniques.

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ABBREVIATIONS

2,4,5-T	:	2,4,5-trichlorophenoxyacetic acid
2,4-D	:	2,4-dichlorophenoxyacetic acid
2iP	:	2-isopentyl adenine
AS	:	Agar + sucrose medium
ANOVA	:	Analysis of variance
BA	:	Benzyladenine
BAP	:	Benzylaminopurine
CH	:	Cameron Highlands
FRIM	:	Forest Research Institute Malaysia
GB5	:	Gamborg B5 Basal Medium with Minimal Organics
GH	:	Genting Highlands
HC1	:	Height category 1 (< 0.5 metres high)
HC2	:	Height category 2 (0.5 - 0.99 metres)
HC3	:	Height category 3 (1.0 - 1.99 metres)
HC4	:	Height category 4 (2.0 - 2.99 metres)
HC5	:	Height category 5 (3.0 - 3.99 metres)
HC6	:	Height category 6 (> 4.0 metres)
IAA	:	indole-3-acetic acid
IBA	:	indole-3-butyric acid
ISTA	:	International Seed Testing Association
MS	:	Murashige and Skoog medium
NAA	:	α-naphthalene acetic acid
NN	:	Nitsch & Nitsch medium
PAR	:	Photosynthetic active radiation
RBG	:	Royal Botanic Gardens, Kew, Richmond, United Kingdom
RE	:	Reproductive efficiency
RH	:	Relative humidity
SH	:	Schenk and Hildebrant medium
UNAM	:	Universidad Nacional Autónoma de México
VW	:	Vacin & Went medium

GLOSSARY

Batch	:	consisting of several lots
Contagious	:	non-random distribution of individuals in a given area
Density	:	number of plants in a unit area
Lot	:	consisting of a predetermined number of seeds
Vulnerable	:	taxa believed likely to move into the endangered category in the near future if the causal factors continue operating. Included are taxa of which most or all the populations are decreasing because of over-exploitation, extensive destruction of habitat or other environmental disturbance; taxa with populations that have been seriously depleted and whose ultimate security is not yet assured; and taxa with populations that are still abundant but are under threat from serious adverse factors throughout their range (definition following the International Union for the Conservation of Nature and Natural Resources Red List Categories)

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 THE PROBLEM

The genus *Nepenthes*, commonly known as the tropical pitcher plants, belongs to the carnivorous group of plants. Its pitchers of different shapes and colours present a unique feature and are of great horticultural attraction. The genus is well documented but mainly in the horticultural context. Basic understanding of its biology is still elusive, making it difficult to assess its conservation status in Malaysia. The problems related to its status are highlighted below.

1.1.1 Endemicity

The centre of diversity of the genus is in the tropical region of the Far East, particularly in the Malay Archipelago, where at least 89% of the total number of species occurs (Danser, 1928; Jebb & Cheek, in press). All except nine species are restricted to this archipelago and most of them are endemic to certain islands (Jebb & Cheek, in press). Some of the endemics have wide habitat distribution while others have a narrower distribution. Outside the archipelago, Madagascar, Seychelles and Sri Lanka have two and one endemic species respectively. Since the level of endemicity is an important criterion used to consider whether a particular species merits conservation attention, this genus clearly merits such consideration.

1.1.2 Trade

Nepenthes spp. is a much sought-after ornamental, particularly in the United States, Europe and Japan. Plants, often obtained from the wild, are propagated and sold at lucrative prices. Many horticulture-based companies undertake and develop breeding programs to produce hybrids; because of their spectacular pitchers (Slack, 1988) they are

traded at much higher prices. Annual trade figures for *Nepenthes* spp. are poorly documented, but market-related surveys indicate that the figures may amount to millions of US$ per annum. A preliminary inspection conducted in 1992 showed that pitcher plants, particularly those that are hybrids and those that are rare or derived from rare parental species, are traded at high prices in the international market (Table 1).

The initial sources of materials are obtained from wild populations occurring in many different sites in the country of origin and/or countries with high genetic diversity. Because of the overwhelming interest generated by lucrative markets and personal interests, these populations have become targets of over-zealous collectors. The indiscriminate manner of collection, and the complete disregard by the collectors of the need to retain a sustainable population in the niche, hasten the loss of genetic diversity in particular populations. Apart from the attempt by the Royal Botanic Gardens, Kew, England, to reintroduce *N. pervellei* into its country of origin *i.e.* Seychelles (Redwood & Bowling, 1990), no other attempts have been made either by enthusiasts or by horticulture-based companies to reintroduce rare or endangered *Nepenthes* sp. into its original habitats. In addition, many countries have experienced cases of unlawful collecting which has adversely affected the local populations. In the face of this threat, Malaysia, as the country having the highest diversity of *Nepenthes* spp., has placed the very spectacular *N. rajah* on the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora, 1975) Appendix 1 and all other species on Appendix 2 so as to safeguard the survival of the wild populations and to monitor the trade (Anon, 1981).

In the preliminary report prepared for the Review of Significant Trade in Plant Species listed in Appendix 2 of CITES (1983-1989), an average of 60.7% and 59.4% of the *Nepenthes* spp. exported from Malaysia and Brunei Darussalam was in the wild form

(World Conservation Monitoring Centre, 1991) (Appendix 1). The large percentage traded in the wild form clearly reflects the need for some form of monitoring the export of planting materials out from the country of origin.

1.1.3 Conversion of its habitats

Apart from trade, another factor that affects the viability of wild populations is the conversion of their habitats into non-forest land uses. Although the genus is found in both lowland and highland habitats, it is most diverse in the montane environment. In Malaysia's perennially hot and humid climate, holiday resorts in the highlands have a tremendous public appeal. The socio-economic pressures and needs have grave implications for the continued existence of the species in sites earmarked for development. In at least one known site, the population size has been reduced in parallel with the fragmentation of the habitats. This phenomenon is becoming common as more hill sites are being developed.

Unlike crop species where an *ex situ* conservation approach has enabled large amounts of germplasm to be kept in a secure manner, such an approach has not been attempted for many of the tropical forest species such as *Nepenthes* spp. Most of the genetic diversity of *Nepenthes* sp. is still confined to the forests and it is not completely secure under the present network system of forest reserves and national parks in Malaysia. The forest reserves, which are far more extensive in area than the national parks, were delineated on the basis of their timber resources and were accordingly given a certain degree of protection (National Forestry Policy 1978, Amendment 1993; National Forestry Act 1984, Amendment 1993). The harvesting practised here is selective, allowing a reasonable amount of regeneration to take place, but in the case of *Nepenthes* spp., the habitats where greatest diversity occurs do not contain timber resources. On the other hand, a piece of the highland may be degazetted for development, with the result that clear-felling leads to

extinction of populations. At present, the network of national parks is seen to be more effective in conserving biological diversity, despite the fact that it is relatively restricted in area.

1.1.4 Lack of biological information

Despite its horticultural interest, surprisingly little is known about the ecology of *Nepenthes* spp. in their natural habitats. Apart from the taxonomic revision of the family by Danser (1928) and several more recent workers (Adam *et al.*, 1989; Jebb & Cheek, in press), no comprehensive work has been conducted to understand the general biology or ecology of any of its species, particularly the endemics with restricted distribution. Most studies on *Nepenthes* sp. have revolved around their 'prey' and the inhabitants of the pitchers. Crucial information needed for conservation purposes, such as that related to the reproductive biology, population dynamics and seed storage, is not available for species endemic to Peninsular Malaysia. One has to bear in mind that in many conservation programmes, the resource managers only have a superficial understanding of the biological diversity that they are supposed to manage and they often have to seek professional advice. In the absence of adequate information, it is not possible to develop effective conservation strategies and implementation measures.

As a partial response to the above threats, the International Union for the Conservation of Nature and Natural Resources (IUCN) has, in 1987, established, under the Species Survival Commission (SSC), a specialist group on carnivorous plants. This group aims to promote the implementation of research, conservation and management programmes, to review the status of carnivorous plants and to develop conservation strategies and projects to be carried out by appropriate organisations.

1.2 THE OBJECTIVES

Nepenthes macfarlanei Hemsl. was chosen as a case study for three reasons. Firstly, it is endemic to Peninsular Malaysia. Secondly, its distribution is confined to the montane areas, which are at present facing development pressures. Finally, apart from its taxonomic status, very little is known about its biology and ecology and the available information is not adequate for the recommendation of measures for conservation.

This study aimed to :

1. document the population density and spatial distribution of *Nepenthes macfarlanei* in selected montane sites,
2. study the phenology and reproductive biology,
3. study the possibility of long-term seed storage by conventional and new techniques (cryopreservation)
4. and mass multiplication by micropropagation techniques as a complement to conservation,
5. recommend practical *in situ* and *ex situ* conservation measures.

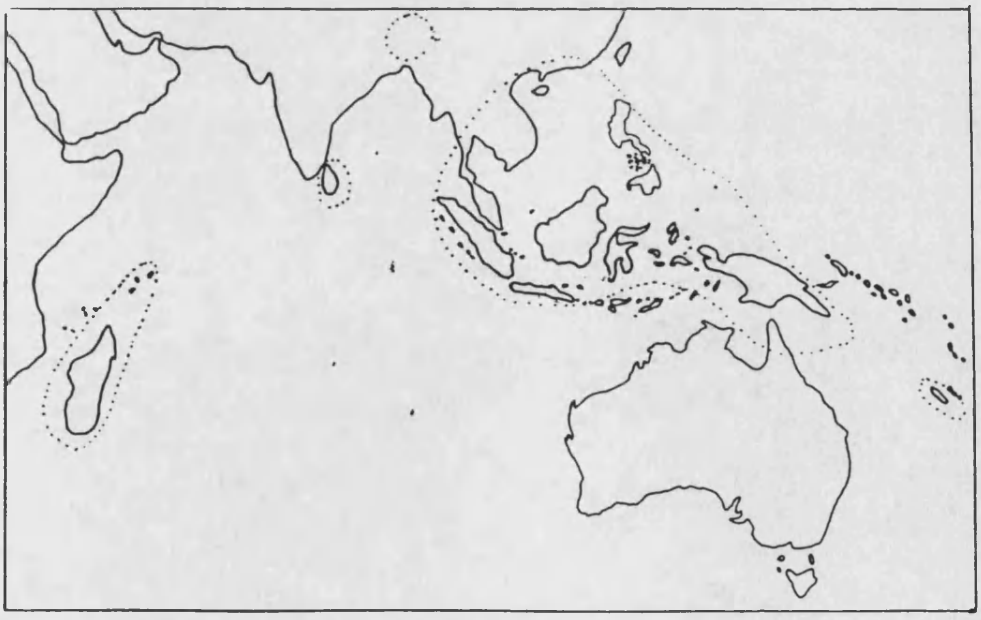
CHAPTER 2

THE GENUS *Nepenthes*

2.1 DISTRIBUTION

The genus *Nepenthes* belongs to the family Nepenthaceae. This family is monogeneric and about 84 species have so far been described (Macfarlane, 1908; Danser, 1928; Harms, 1936; Jebb, 1991; Jebb & Cheek, in press). Its distribution extends from Madagascar and Seychelles in the west, northwards to the Khasi highlands in India and Southern China, eastwards to New Caledonia and to York Peninsula in Australia (Fig. 1). The centre of diversity is in the Malay Archipelago which encompasses Indonesia, Malaysia, Singapore, Thailand and Brunei and at least 75 species (89% of the total number of species) occur here (Danser, 1928; Jebb & Cheek, in press).

Fig. 1 : The phytogeography of *Nepenthes* spp.



This genus has a large number of endemic species, the majority of which are confined to Malay Archipelago. The island of Borneo is particularly rich in species diversity and it has at least thirty species, twenty one of which are endemic (Danser, 1928). Most of them have evolved localised habitat preferences, some of which are narrow while others are broad. The hyper-endemics are believed to occupy only a single locality. Danser (1928) remarked that no less than 25 out of 65 species in Borneo were known from only one location, while Smythies (1963) mentioned that almost one-third of the species in the genus are similarly restricted in their distribution. Recent studies by Adam *et al.* (1989) in Borneo showed that at least 10 species occur only in single localities. This represents 14% of the total number of taxa. Examples of such species are *N. northiana*, *N. muluensis*, *N. villosa*, *N. boschiana* and *N. campanulata*. *N. northiana* and *N. muluensis* are known only in Bako and Mt. Mulu (Bako and Mulu National Parks, Sarawak) respectively, *N. villosa* is known only on Mt. Kinabalu (Kinabalu National Park, Sabah) while *N. boschiana* and *N. campanulata* are known only on Mt. Sakoembang and Mt. Ilas Bungean (Kalimantan) respectively. Another example of a hyper-endemic is *N. paniculata* which is known from only a single collection from Doorman Top, Papua New Guinea (Jebb, 1991). Examples of broad endemics are *N. bicalcarata*, *N. hirsuta*, *N. lowii* and *N. veitchii*. *N. bicalcarata*, *N. hirsuta* and *N. veitchii* are fairly widespread species in Borneo while *N. lowii* has been collected from ten mountain summits (Adam *et al.*, 1989). In Peninsular Malaysia, there is no narrow endemic; the three endemic species *i.e. N. macfarlanei*, *N. sanguinea* and *N. gracillima* are found in most mountain ranges (Danser, 1928). *N. gracillima* is more restricted compared to *N. macfarlanei* and *N. sanguinea* and, at present, this species is known from only seven mountains *i.e.* Mt. Tahan, Mt. Tapis, Cameron Highlands, Fraser's Hill (all situated in the state of Pahang), Mt. Stong (Kelantan), Mt. Padang (Trengganu) and Mt. Ulu Kali (Selangor) (Kiew, 1990). The most widespread species of the genus is *N. mirabilis*, followed by *N. ampullaria* and *N. maxima*. *N. mirabilis* can be found from Southern China

to Philippines and Australia while *N. ampullaria* and *N. maxima* are distributed from Sumatra to Papua New Guinea and Borneo to Papua New Guinea respectively (Danser, 1928, Kurata, 1976; Adam *et al.*, 1992).

The high levels of endemism in the genus merits some form of protection and priority should to given to hyper-endemics. In Peninsular Malaysia, only one mountain range *i.e.*, Tahan Range is protected under the national park system. Other mountain ranges have been placed under the network of forest reserves. In Sabah & Sarawak, the habitats of all the hyper-endemics are protected under the network of national parks; in addition, the species are afforded complete protection under the state ordinances. There is a disparity between the types of forest habitats and sizes placed under the national park systems in Peninsular Malaysia, Sabah and Sarawak. In addition, unlike Sabah & Sarawak, the states in Peninsular Malaysia do not have enactment for the protection of particular plant species of ecological or economic importance. As far as *Nepenthes* spp. are concerned, however, this disparity is not critical as most of the hyper-endemics are found in Sabah & Sarawak. It is not known whether the hyper-endemics occurring in other countries have been accorded some form of protection.

2.2 HABITAT DISTRIBUTION

In Malaysia, *Nepenthes* spp. can be found from sea level to mountain tops. They are confined to the edaphic climaxes of poor soils such as sandy coasts (Kurata, 1976; Phillips & Lamb, 1988) (*e.g. N. albo-marginata*), peat swamp forests (Kurata, 1976; Shivas, 1984; Phillipps & Lamb, 1988) (*e.g. N. ampullaria*, *N. bicalcarata* and *N. rafflesiana*), heath forests (Ashton, 1971) (*e.g. N.x trichocarpa, N. albo-marginata* and *N. gracilis*), limestone forests (Smythies, 1963; Anderson, 1965; Kurata, 1976) (*e.g. N. northiana, N. muluensis, N. albo-marginata* and *N. mapuluensis*) and montane ericaceous (mossy) forests

(MacFarlane, 1908; Danser, 1928; Smythies, 1963; Kurata, 1976; Shivas, 1984; Phillipps & Lamb, 1988; Jebb, 1991). They are frequently found in ultrabasic soils (Phillipps & Lamb, 1988; Adam *et al.*, 1989) (*e.g. N. veitchii, N. macrovulgaris* and *N. burbidgeae*) and in open, degraded sites (*e.g. N. gracilis, N.x trichocarpa, N. reinwardtiana, N. rafflesiana, N. x hookeriana* and *N. mirabilis*) but they rarely occur in high-canopied alluvial lowland or hill forests such as the dipterocarp forests (Holttum, 1940a; Green, 1967; Chai & Radcliffe, 1984; Hotta & Tamin, 1986). However, Smythies (1963) reported that *N. bicalcarata* is common in high-canopied peat swamp (*Shorea albida*) forests in Sarawak (Borneo) while *N. gracilis* and *N. rafflesiana* are common in the heath forest (also of *Shorea albida* forest, but the trees are much reduced in size). In addition, Adam *et al.* (1992) reported that *N. ampullaria, N. albo-marginata* and *N. rafflesiana* are occasionally found in gaps of high-canopied lowland forests. Smythies (1963) mentioned the rare presence of *Nepenthes* sp. in submontane/lower montane oak laurel forests but Adam *et al.* (1992) reported that many species, *e.g., N. tentaculata, N. hirsuta, N. burbidgeae* and *N. lowii* can be found in this forest formation.

Smythies (1963) postulated the unfavourable physical environment for the lack of distribution in high-canopied forests. He, including Lim & Prakash (1973) and Corker (1986) suggested that the light factor is a significant influence to the establishment and development of the species in the forest. If it does influence the growth of *Nepenthes* seedlings, it would be clearly reflected in the first instance, in the comparative abundance (and growth dimensions) of individuals in different stages of development in open sites and canopied forests. However, past reports made no mention of these. Even though large gaps frequently occur in high-canopied forests and these will provide sufficient light, the edaphic factors may restrict the growth of the plant. In a related observation, Green (1967) found that shade can influence the development of the tendril and pitcher. Similarly,

Mazrimas (1979) noted that different light intensities influence the growth rate and pitcher production in *Nepenthes*.

To the above postulations, it is possible that there is a more fundamental issue preceding the physiological factors. Plants regenerate either from seeds or other propagules already present in the soil, or in the case of gaps, from seeds or propagules entering the site. In the case of *Nepenthes* spp., the absence of populations in the lowland canopied forests means that its species can only regenerate in gaps when seeds enter the site from forest fringes. However, since the seeds are wind-dispersed, any encounter with a closed canopied forest will obstruct the movement and reduce the distance of seed dispersal. Due to the forest stratification, the seeds may land not only on ground but on other stratum as well. Here, the physiological factors such as the need for sufficient light and water come into play.

The species and genetic diversity are greater in elevations covered by mossy forests (MacFarlane, 1908; Danser, 1928; Smythies, 1963; Kurata, 1976; Shivas, 1984; Phillipps & Lamb, 1988; Jebb, 1991) and for a tropical taxon, some of the species are remarkably adapted to conditions in high altitude ecosystems. These montane species are distributed locally and never found in low elevations. Examples of such species in Peninsular Malaysia are *N. macfarlanei*, *N. sanguinea* and *N. gracillima* while in Borneo are *N. lowii*, *N. rajah*, *N. veitchii* and *N. edwardsiana*. This is quite unlike the lowland species where at least seven are known to occur from sea level up to 1000 metres (Kurata, 1973) but higher up, they disappear. Examples of such species are *N. rafflesiana*, *N. ampullaria* (personal observations) and *N. albo-marginata* (Shivas, 1984). All the lowland species found in Malaysia are present elsewhere in the Malay Archipelago.

The phenotypic variability of the plants seems to be higher in the montane species than those found in the lowlands and this is often conspicuously reflected in the colour of the pitcher. For example, three dominant colours of the pitcher have been found in different populations of *N. gracillima.* These plant populations have been found on different summits: on Mt. Ulu Kali (Pahang), the pitchers are black or rice green while on Mt. Tahan (border between Pahang & Kelantan), the pitchers are ivory white with rosy spots or deep-purple black, and on Mt. Stong (Kelantan), they are deep red-purple with white flecks (Kiew, 1990). Wide variation in the size of pitcher and inflorescence has also been observed in this species (Ridley, 1915; Kiew, 1985). In *N. macfarlanei* and *N. sanguinea,* however, the colour and shape of the pitchers are less variable (personal observations) although in *N. macfarlanei,* dimorphic colours of the pitchers frequently occur.

2.3 HISTORY

Nepenthes was first recorded by E. de Flacourt, a colonial governor of French Madagascar in 1658 under the name of *Amramitico.* This species was later described as *N. madagascariensis* by J.L.M. Poiret in 1797. Later discoveries in Sri Lanka brought forth new species such as *Planta mirabilis distillatoria* (later published as *N. distillatoria* L.) (Linnaeus, 1753) and *Nepenthes zeylanicum flore minore* (Nepenthes of Sri Lanka with small flower) (Kurata, 1976).

Its common name, pitcher plant, arises from the pitcher-like containers hanging by the tendrils developed from the tip of leaves. Its generic name, *Nepenthes,* is said to be associated with the Greek word *νηπενθης* which means "banishing sorrow". This word was used to describe an incident in the epic Odyssey where Helen threw a drug into the wine which was supposed to free men from grief, anger and all ills. This word has two interpretations *i.e.* a certain drug or anaesthetic capable of banishing one's grief or a

container for such purposes *i.e.* a medicine bottle (Kurata, 1976). The generic name was first used by J.P. Breyne in his publication on exotic plants (1689) and was later adopted by Linnaeus. The vernacular names used in Malaya, Borneo and Sumatra are also based on its pitchers. People of Malaya and Borneo refer it as "periuk kera" (monkey rice pot) (Burkill, 1935), while in Sumatra, "tahul-tahul" (small kettle) and "kantong samar" (sack of semar (a clown in the shadow play)) are commonly used (Heyne, 1927).

The phytogeography of the genus and the disjunct distribution on islands at the western part of its phytogeography are believed to be related to the continental drift that took place during geological times.

2.4 MORPHOLOGICAL DESCRIPTION

N. macfarlanei is a dioecious liana. The family is distinguished from other groups of plants by the presence of pitchers hanging by the tendrils extending from the leaves. The stems are erect or prostrate in short shoots later becoming long and climbing. The length range from a rosette form to more than 5 metres. Saplings have short but distinct internodes, later elongating up to 18 cm in length. The stem is either cylindrical or obtusely angular. *N. macfarlanei* may occur either as epiphytic or terrestrial plants (Smythies, 1963).

The leaf is composed of a phyllodium, tendril, pitcher and lid. The phyllodium of climbing stem is sessile, thinly coriaceous, entire, oblong to lanceolate to spathulate, 5-18 x 2-5.5 cm in size with a rounded or slightly cordate and semi-amplexicaul base. Nerve arrangement is pennate and parallel; the pennate nerves are indistinct while the longitudinal ones are distinct. The midrib is robust and prominent below, prolonging beyond the lamina as a thick, strong tendril. The tendril is 2 to 4 times as long as the phyllodium and has a curl at

about two thirds of its length. In short shoots and rosettes, the phyllodium is larger than the tendril and the tendril often do not curl (Danser, 1928).

Pitchers are borne along the length of the plant and they are dimorphic at different levels (Veitch, 1897; Danser, 1928; Kurata, 1976; Jebb, 1991). Ground pitchers are smaller and widest near the base while lower pitchers are shortly incurved, infundibuliform in the lower part, slightly ventricose in the middle and cylindrical in the upper part (Plate 1a). The ground and lower pitchers have fimbriated wings (MacFarlane, 1908; Danser, 1928; Holttum 1940a). The upper pitchers are infundibuliform and somewhat contracted under the mouth (Plate 1b), here the wings are reduced to prominent ribs. The pitcher may attain 20 cm in length. In rosette and juvenile plants, most leaves produce pitchers (personal observations). Smythies (1963) and Kiew (1990) observed that plants may grow vigorously without producing pitchers and in mature climbing individuals, tendrils more often function as climbing aids and do not produce pitchers. On Mt. Ulu Kali (Pahang), only 38% of the leaves of *N. macfarlanei* produced pitchers while in *N. gracillima,* 66 % of the leaves produced pitchers. At any particular time, only one pitcher per plant and two pitchers per plant were available for *N. macfarlanei* and *N. gracillima* respectively (Kiew, 1990).

The mouth is horizontal, incurved and elongated into a short neck towards the lid. At the rim is a peristome which is flattened and ribbed on its upper surface. The teeth of the inner margin of the peristome are 3 to 6 times as long as broad. The lid is suborbicular to slightly cordate with numerous large-rimmed glands and long thick bristles on the lower surface (Hemsley, 1906; Macfarlane, 1908). The lid covers the pitcher when young but opening later (Danser, 1928). In rosette pitchers, the inner lower surface is glandular with overarching glands, making the outer surface look minutely bullate. In upper pitchers, with

Plates 1a - b : Pitchers of *N. macfarlanei*

Plate 1a : Lower pitcher

Plate1b : Upper pitcher


1a


1b

the exception of a narrow part under the peristome, the entire inner surface is shining and glandular. The glands are usually overarched by the protrusion of parenchyma in the pitcher wall. Like many species, there is a spur near the lid. This spur is believed to be the true leaf apex (MacFarlane, 1908; Lloyd, 1942 and Schmid-Hollinger, 1970).

Previously, many people erroneously considered the pitcher to be a reproductive organ. This misconception was corrected by Griffith (1843) and Hooker (1859). Griffith *loc.cit.* observed that pitchers are modifications of the excurrent midrib of the leaf. Later, Hooker in his 1859 account on the origin and development of pitchers in *Nepenthes*, clearly showed that the pitchers are modifications of a gland situated at the apex of the midrib of the leaf and that pitcher development is an organised process. In *N. gracilis*, the first stage of pitcher development consist of a minute, blunt conical body with a slight depression and a shallow oval cavity. The cavity is a subterminal gland and it represents the future pitcher. The entire structure is recognised by its epidermal layer of imbricately, disposed glassy cells. The pitcher continues to grow in all planes into an adult size (Dickson, 1883). Observations on *N. villosa* in Mt. Kinabalu (Sabah) showed that the development of the leaf tip into a fully mature, just opened pitcher takes an average of 8-10 months (Phillipps & Lamb, 1988). Pitchers are readily formed even in small seedlings and they are often more conspicuous than the lamina of the leaf (personal observations).

Beaver (1983) reported that pitchers of *Nepenthes* sp. may persist for 6-7 months with a maximum life span of one year. Kiew (1990), in her study on *N. gracillima* and *N. macfarlanei*, reported an average life span of about 4 months with a range of 2.5 - 7.5 months while Phillipps & Lamb (1988) reported, for *N. villosa*, a life span of 8-10 months.

2.5 NATURAL HYBRIDS

The occurrence of natural hybrids is often reported in *Nepenthes* spp. Phillipps & Lamb (1988) and Adam *et al.* (1989, 1992) have documented the hybrids found in Sabah & Sarawak (Borneo). For example, a natural hybrid of *N. lowii* and *N. edwardsiana* (*N x trusmadiensis* Marabini) was discovered in 1983 by Marabini from Mt. Trusmadi, Sabah at an elevation of 2625-2900m (Briggs 1984). Its peristome was a combination of the deeply ribbed peristome of *N. edwardsiana* and the almost absent peristome of *N. lowii*. The top part of the pitcher resembled that of *N. lowii* while the lower half resembled that of *N. edwardsiana*. It has the largest pitchers after *N. rajah*. The population is small and the hybrid is not known elsewhere. So far, 18 hybrids have been reported from the Malay Archipelago (Phillipps & Lamb, 1988; Adam *et al.* 1989, 1992).

Variability in the pitchers has caused considerable taxonomic confusion. Many previous collections of *Nepenthes* spp. did not take into account the dimorphy character and the variability in pitchers. Herbarium collections were often incomplete representatives and this has often led botanists into misidentification. The fairly common occurrence of hybrids indicates that introgression and hybridisation occurs with ease in the genus and that there is an apparent lack of sporophytic and gametophytic incompatibilities. Although this will undoubtedly have increased the gene pools and genetic variability of conspecific populations, it is not known whether such hybrids are fertile and their population self-sustaining. Many of the natural hybrids are hyper-endemics with small population size. Selection pressures such as natural selection, genetic drift, mutation and man-induced pressures are likely to favour individuals which are more vigorous and fecund. Danser (1928) remarked on the lack of information concerning the fertility of natural hybrids, the ploidy levels and the survival rates of their progenies. Until today, such information is still not available.

2.6 FAUNA IN THE PITCHER

2.6.1 The functions of the pitcher

The *Nepenthes* pitcher is wonderfully adapted to trapping insects which provides the plant with nutrients, particularly nitrogenous compounds. The pitcher has three functions which are trapping, decomposing and digesting prey organisms into amino acids and inorganic nutrients and absorbing them (Thienemann, 1932, 1935; Lloyd, 1942). For the plants to enhance the rate of nutrient absorption, there are three strategies : to maximise the rate of nutrient input into pitchers, to maximise the rate of decomposition and digestion and to minimise the loss of nutrients (Kato *et al.*, 1993). Prey trapping is the only means of nitrogenous inflow into a pitcher. This function is aided by the secretion of nectar and the smooth inner surface of the pitcher. According to Heslop-Harrison (1978), Givnish *et al.* (1984) and Janzen (1985), the plant is able to provide sufficient amounts of nectar, which consists mainly of sugars, because they grow in moist and sunny habitats where water supply and photosynthetic energy are not limited and the production of carbohydrate is relatively cheap. The inner surface of pitcher can be partially or wholly glandular, depending on the species (Danser, 1928). Under the electron microscope, the smooth zone is composed of a dense coating of minute readily detachable wax scales which prevents any organism from clinging to the wall (Juniper *et al.*, 1989).

2.6.2 The attraction by the pitchers

In carnivorous pitcher plants, the shape and colour pattern, together with tactile and olfactory stimuli, are believed to form guiding signals for insects (Wiens, 1978; Slack, 1979; Joel, 1983; Joel *et al.*, 1985; Joel, 1986 and Stowe, 1988). In most *Nepenthes* sp., the pitcher is "beautifully coloured with chocolate, garnet or dark red" (Slack, 1979). Steiner (1948) and Joel *et al.* (1985) argued that the dark patches of the pitcher mimic dark spots which are known to attract flies. In addition, the secretion of nectar by the lid,

peristome and fimbriated wings of the pitchers serve, together with visual and olfactory cues, as an attractant tempting insects to reach and forage until some of them accidentally stumble and fall into the digestive cavity (Delpino, 1874; MacFarlane, 1893; Holttum, 1940a; Lloyd, 1942; Wickler, 1968; Heslop-Harrison, 1978 and Williamson, 1982). In most species, the finely-grooved peristome may serve as guiding trails (Dickson, 1883; Danser, 1928). The fimbriated wings running down the front of the pitcher may also serve as a guide for insects to climb up from the ground to the mouth of the pitcher (Phillips & Lamb, 1988).

2.6.3 Prey

Ants were the predominant group of insects found in the pitchers of *N. albo-marginata, N. ampullaria, N. gracilis* (Beaver, 1979), *N. alata, N. mirabilis, N. reinwardtiana, N. spathulata* (Kato *et al.*, 1993), *N. bicalcarata, N. rafflesiana, N. macrovulgaris, N. rajah, N. lowii* and *N. tentaculata* (Dover *et al.*, 1928; Thienemann, 1932; Adam *et al.*, 1989). In an attempt to describe the aggregated distribution of ants in the pitchers, Joel (1988) suggested that other chemicals, such as trail pheromones of ants which successfully returned to their nests with the nectar, may participate in the process of prey attraction.

Apart from ants, apterous termites have also been recorded (in *N. albo-marginata)* but since termites are not attracted to nectar, Kato *et al.* (1993) suspected that there may be a secondary attraction present in the pitchers. This may be related to the presence of humus in some pitchers, as observed by Phillipps & Lamb (1988) in the pitchers of *N. bicalcarata.* Other invertebrates found in pitchers included Phalangida, Blatteridae (in *N. spathulata*), saprophagous arthropods and midges (in *N. bongso*). Kato *et al.* (1993) suggested that the colour of the peristome mimics rotting substances and in older pitchers, the fluid releases a putrefaction odour which attracts arthropods.

2.6.4 Inhabitants

The pitcher does not consume all its visitors; in fact pitcher morphologies are believed to be related to both 'prey' capture and assemblages (Kato *et al.*, 1993). The 'prey' function is often disturbed or supported by other organisms whose tolerance to hydrolytic enzymes enabled them to inhabit the pitcher. Trapped organisms are degraded and decomposed by micro-organisms and are digested by the enzymes secreted from the pitcher itself (Nakayama & Amagase, 1968; Amagase *et al.*, 1969; Tokes, 1974) and/or from the organisms (Juniper *et al.*, 1989). Insect inhabitants of the pitcher have been believed to be opportunists because the adults fly away from the pitchers after having utilised its nutrients for growth and development (Beaver, 1979).

Direct observations by scanning electron microscopy on *N. macfarlanei*, *N. sanguinea* and *N. mirabilis* showed that micro-organisms such as bacteria and yeasts (particularly *Cryptococcus albidus* and *Tilletiopsis sp.*) were abundant around nectaries. They were found in the crevices of nectar glands on the lower surface of the lid, in the epidermal grooves and depressions on the outer surface of the pitcher wall and on the terraced ridges of the peristome (Shivas & Brown, 1989). This cluster indicated that nectar secretions are a source of nutrients. Shivas & Brown *loc. cit.* demonstrated that the diversity of yeast varied between the upper and lower pitchers of *N. macfarlanei* and *N. mirabilis*. Seven species of yeasts were found in the upper pitchers of *N. macfarlanei* while three species occurred in *N. mirabilis*. Lower pitchers supported significantly higher populations of yeast than upper pitchers. This could be due to rain-splash which introduces soil-borne inocula into the pitchers. Apart from yeast and bacteria, blue-green algae have also been found in some pitchers and Kiew (1990) suggested that they may contribute nutrients through their nitrogen-fixing ability.

Other assemblages include the filter and detritus feeders. These feeders search for food at different levels in the pitcher (Beaver, 1979). Filter feeders such as culicid larvae in the genera Tripteroides, Culex and Uranotaemia sieve off fine particles, bacteria and protozoa in the fluid and on the surface of dead insects. The detritus feeders such as the ceratopogonids *Dasyhelea* and *Zwickia* forage for detritus at the bottom of the pitcher (Hepburn, 1918; Beaver, 1979). The browsing of prey by these feeders is believed to accelerate the rate of decomposition but, according to Addicott (1974), this may not occur as filtration will reduce the density and diversity of micro-organisms responsible for the processes. Steffan & Evenhuis (1981) have demonstrated that the *Toxorhynchites* larvae is a effective predator of detritus feeders.

The nectar and the teeming life in the pitcher are also robbed and predated by larger invertebrates. In lowland species such as *N. gracilis, N. mirabilis* and *N. ampullaria*, the groups of insects most commonly encountered were Diptera (particularly Culididae) (Beaver, 1979) and ants (Adam *et al.*, 1989; Kato *et al.*, 1993). In highland species such as *N. rajah, N. lowii, N. villosa* and *N. muluensis*, groups trapped were more diverse with ants being less encountered (Adam *et al.*, 1989). Groups trapped included the Formicidae, Coleoptera, Arachnida, Orthoptera, Hymenoptera, Chiloptera, Heteroptera and Dictyoptera. Mosquitoes are common inhabitants in the pitchers (Beaver, 1979) but the only species significant to man is *Aedes albopictus*, a distance relative of *Aedes aegypti*, which is the vector for dengue in urban areas (Khoo, pers. comm.). Fortunately, the pitcher forms a only minute fraction of its breeding sites (Beaver, 1979).

In addition to insects, toads (*Pelophryne brevipes*) and tadpoles of several frogs such as *Kalophrynus* sp., *Megophrys aceras*, *Philautus vermiculatus* (Kiew, 1990), *P. mjobergii* (Phillipps & Lamb, 1988) and *P. aurifasciatus* (Yong *et al.*, 1988; Kiew, 1990; personal

observations) have been found in the pitchers *of N. macfarlanei, N. villosa* and *N. sanguinea.* The Noctuid moth *Eublemna rada* has been reported pupating in the upper pitchers of *N. rafflesiana* in Singapore (Dover *et al.*, 1928) and *Geosesarma malayanum*, a small red crab, has been found in the pitchers of *N. ampullaria* in Endau-Rompin, Pahang (Ng & Lim, 1987). Snail eggs have also been found attached to the upper surface of fresh pitchers in *N. edwardsiana* (Phillipps & Lamb, 1988). Awang *et al.* (1986) recorded red-crab spiders (*Misumenops nepenthicola*) in the lower pitchers of *N. rafflesiana* and *N. gracilis.* Apparently, this spider spins a light web just below the peristome to trap insects and to allow it to move about the slippery walls. Their observation agrees with that of Bristowe (1931) who elaborately described its predatory activities in the pitchers of *N. gracilis* and *N. rafflesiana.* The largest creatures found in pitchers were drowned rats, discovered by Low and St. John in 1858 and more recently by Phillipps and Lamb (1988) in *N. rajah.*

Although most observations have recorded some form of inhabitants/prey, this does not imply that all pitchers will contain them. Ridley (1915) observed that most of *N. gracillima* pitchers on Mt. Tahan were dry and empty of insects. Similarly, Kato *et al.* (1993) discovered that pitchers of *N. bongso* did not contain living inhabitants. They suggested that the fluid may be toxic and organisms are digested entirely by its enzymes.

2.6.5 Fauna relationship

Members of the Nepenthaceae are generally thought to have developed deceptive mimetic systems, which enable them to deceive insects by attracting them into traps (Darwin, 1875; Heslop-Harrison, 1978; Williamson, 1982). They bear flower-like appendages, advertise visual and olfactory signals and share features of flower models. These features attract insects which have innate floral preference or provision experience with flower models, but

little or no experience with the mimic (Williamson, 1982). Wickler (1968) and Wiens (1978) classified carnivorous traps into the group of aggressive mimicry; more recently Pasteur (1982) suggested aggressive semi-abstract homotypy. Joel (1988) demonstrated that *Nepenthes* sp. are mutualistic with certain insects because both benefit from the food provided. Like ants, such insects will return to the same pitchers or to similar pitchers if they have gained some profit during their first visit. They will rapidly learn that the visual and olfactory characteristics of pitchers lead them to a reliable source of nectar.

2.6.6 The availability of nutrients

Studies have shown that the availability of nutrients derived from prey, as measured from the number of prey in a pitcher, is not necessarily high. In the Papua New Guinean *N. mirabilis* the average number of prey per pitcher was 140 in the lower pitcher and 41 in the upper pitcher, of which more than 80% were ants (Jebb, 1991). On the assumption that a pitcher lives for about six months, Kiew (1990) calculated that these figures represent less than one insect a day per ground pitcher and one insect every 4 to 5 days for the upper pitchers. This quantity of prey and the low number of pitchers indicate that daily capture can be low and that their demand for nitrogenous compounds is not high. Beaver (1985) reported that the food webs in pitchers were more complex at the centre of diversity than at the periphery of its range, but this was refuted by Kato *et al.* (1993) whose work on Sumatran *Nepenthes* sp. showed otherwise.

2.7 USES

Apart from the horticultural interest (see 2.8), little is known about other possible uses for *Nepenthes* spp. *N. rafflesiana* roots have been used to extract several napthoquinone compounds but there was no mention of the potential uses of this quinone (Rizzacasa & Sargent, 1987). The local communities in the Borneo, Sumatera and Malaya utilised the

stem of *N. ampullaria* for rough cordage while roots were used to treat stomach aches, dysentery, coughs and fever (Danser, 1928; Burkill, 1935). In Borneo, the plant is occasionally used as an emetic in cases of poisoning while in Brunei, the digestive juice from unopened pitchers were used as eye-drops and hair oil. The pitcher was once documented as being used in exorcism exercises (Burkill, 1935). In China and Hong Kong, a decoction of *N. mirabilis* is a remedy for high blood pressure and urinary infection. *N. rafflesiana* pitchers were sometimes used to cook cakes or rice and it is said to give an added flavour (Burkill, 1935). The practical use of pitcher and its contents were described by an expedition team to Mt. Tambuyukon in the Crocker Range of Sabah, Borneo. Due to the scarcity of water, the team had to cook their meals using the water contained in the pitchers of *N. edwardsiana*. According to them, tea made from the water had a curiously smoky taste (Briggs, 1984).

2.8 HORTICULTURAL TRADE

In the preliminary report prepared by the World Conservation Monitoring Centre (1991) for the Review of Significant Trade in Plant Species listed in Appendix 2 of CITES (1983-1989), *Nepenthes* spp. were placed in the 44th rank of plants with an annual average trade volume exceeding 1000 plants. The average trade volume over the period of 1983-1989 was 1,168,000 plants. The net trade volume increased 24 times between 1987 and 1988, *i.e.* from 188,000 to 4,626,000 but dropped to 1,011,000 in 1989. No reasons were given for this reduction. Most of the species traded could not be identified accurately and were grouped under *Nepenthes* sp. Where identifications have been made, the more popular ones traded are *N. merilliana, N. mirabilis, N. philippinensis, N. petiolata, N. truncata* and *N. rafflesiana* (World Conservation Monitoring Centre, 1991).

The largest exporter of carnivorous plants (*Nepenthes* spp., *Sarracenia* spp. and *Cephalotus* spp.) is the United States. Over the period of 1983-1989, it exported 61,775 of such plants and less than 50% of these were derived from artificial propagation. The next largest exporter is the Netherlands. Over the same period, it exported 3355 plants but all were derived from artificial propagation. Other countries where more than 90% of the carnivorous plant trade was conducted with artificially-propagated plants include Australia, Philippines, France, Canada and Germany (in descending order of the total volume exported) (World Conservation Monitoring Centre, 1991) (Appendix 1). The low percentage of artificially-propagated plants exported from United States raises the question of where the live materials of the carnivorous species were initially obtained. One has to bear in mind that the trade trends published under CITES are not entirely accurate as many cases go unmonitored or unreported by member countries. In addition, there are other nations who partake in trade but are not parties to the Convention and are therefore not obliged to comply with its guidelines. The actual trends and figures are still elusive.

The report on 'The Wild Plant Trade in Europe - Results of a TRAFFIC Europe Survey of European Nurseries'(Jenkins, 1993), stated that :- "outside Europe, demand for *Nepenthes* is particularly high in Japan and United States and that stocks of wild plants have been depleted to supply these markets. Most plants sold in Europe are hybrids, with annual production varying from 3000 to 20,000. Specialist dealers in Europe offer a number of named forms, some of which are species, others varieties or hybrids. Prices may be relatively high for rare forms (*e.g.* US$65 for *N. eymaiae*, N. *spectabilis* and *N.* "Kondo Pectinata" in one German catalogue, with up to US$215 reported for some species). It is unclear whether any of those offered for sale in Europe are wild-collected." In the compilation of catalogues produced by major nurseries in the United Kingdom, Europe, United States and Japan, the author noted that prices vary with species and countries with

the most expensive being *N. tentaculata* and *N. maxima* sold in Japan (Table 1). Hybrids are generally cheaper than species. Because propagation by seed is hampered by the lack of availability, the rapid loss of viability and low seedling establishment rate (Corker, 1986), much of the original parent material could have been wild plants. Even though no conclusive information was available on the proportion of *Nepenthes* plants traded under the group of carnivorous plants, Malaysia and Brunei Darussalam exported at an average of 60.7% and 59.4% of carnivorous plants respectively between 1983-1989. Since only one carnivorous plant genus has its centre of diversity in the Malay Archipelago, the plants that are most likely involved are the *Nepenthes* species. In addition, since artificially propagated plants comprised only 39% and 41% of the volume traded by Malaysia and Brunei Darussalam respectively, the rest must have been the wild forms or pieces of the wild forms (World Conservation Monitoring Centre, 1991).

Table 1 : Price range for *Nepenthes* sp. in the international market (as of 1993)

Species	US Dollars
N. tentaculata	31-304
N. maxima	304
N. lowii	71
N. gracillima	67
N. pectinata	67
N. spectabilis	67
N. madagascariensis	30
N. bicalcarata	29
N. khasiana	25
N. bicalcarata x gracilis	25
N. rafflesiana x gracilis	25
N. mirabilis x hookeriana	10

CHAPTER 3

THE POPULATION DYNAMICS OF *N. macfarlanei*

THE POPULATION DYNAMICS OF *N. macfarlanei*

3.1 INTRODUCTION

N. macfarlanei is endemic to Peninsular Malaysia. It occurs only in the montane ericaceous forests (MacFarlane, 1908; Danser, 1928; Smythies, 1963; Kurata, 1976; Shivas, 1984; Phillipps & Lamb, 1988; Jebb, 1991). This forest type occurs at high altitudinal elevation and is characterized by peat soil, cloud cover and an abundance of lower plants. This species is never found in lower elevation forest formations.

Previous botanical surveys indicated that the species occurs in the Titiwangsa, Bintang and Tahan Ranges but is not known to occur in the Timur Range, situated at the eastern part of Peninsular Malaysia (Appendix 2). The species is most widespread in the central and southern parts of the country. To date, only one locality in the northern part of the peninsular has been recorded, which is Mt. Stong (Kelantan) situated close to the eastern border with Peninsular Thailand. However, the species status of the specimen is considered doubtful by taxonomists.

While many peaks has been surveyed from the Titiwangsa Range, only sparse collections were done at other ranges. It is not known how widespread is the occurrence of the species in smaller ranges and how it is distributed within a range, given the appropriate habitat and climatic environment. In addition, it is not known whether the species occurs on the several isolated peaks found in Peninsular Malaysia and its surrounding islands. The present study aims to address these issues by documenting its population density, pattern and phytogeography.

3.2 MATERIALS AND METHODS

3.2.1 Population density and spatial distribution

3.2.1.1 The study sites

3.2.1.1.1 Cameron Highlands

The first study site is located at Cameron Highlands (Lat. 101° 25'E; Long. 4° 30'N) which is about 230 km from Kuala Lumpur, the capital city of Malaysia (Fig. 2). The area straddles the Bukit Kinta Forest Reserve in the state of Perak and the Batu Gangan Forest Reserve in the state of Pahang (Plate 2) (Dept. of National Mapping, 1990). It was selected as the site of study for the following reasons :

(a) The vegetation is characteristic of a virgin upper montane forest,

(b) The site is conveniently accessible by road and well-maintained tracks,

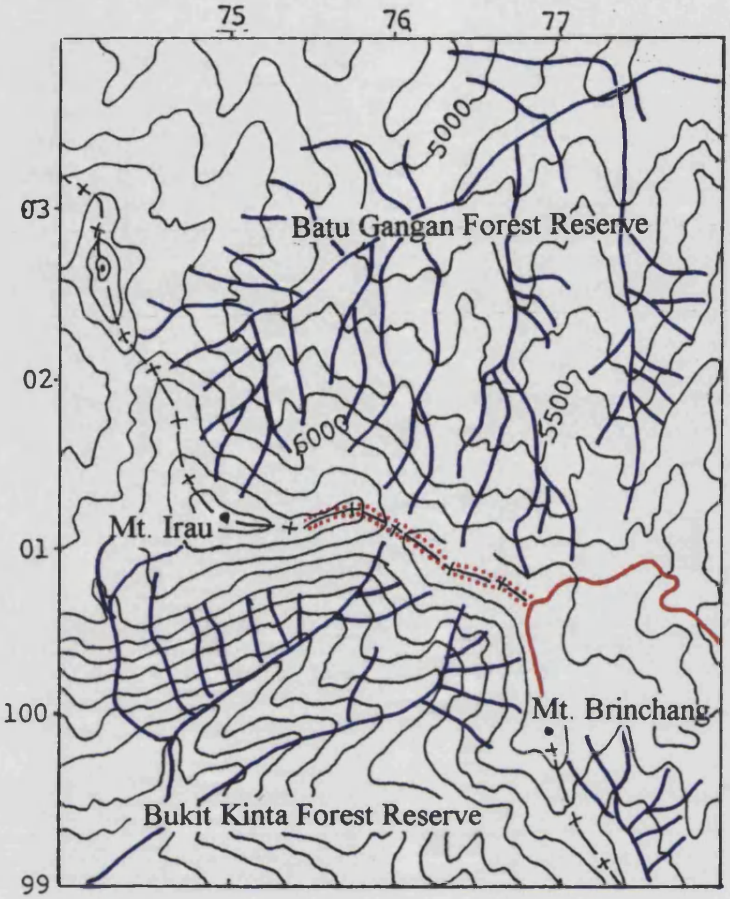
(c) A meteorological station situated nearby provides continuous meteorological data.

The site forms part of the Titiwangsa Range, which is the largest mountain range in Peninsular Malaysia. The terrain is for the most part steep, rising to an elevation of between 1798 and 2072 metres above sea level (Fig. 3) (Dept. of National Mapping, 1990). The trail in which the plots were based is confined to a narrow ridge for approximately one kilometre before continuing into a steep ascent (Plate 2). The area is drained by the Kampar and the Ichat rivers. The mean monthly rainfall is 229 mm with a mean temperature of 17.7 °C and mean relative humidity of 91.1% (Fig. 5a). The wettest and driest periods of the year are September to December and January to February respectively (Malaysian Meteorological Service, 1992-1994). Much of the area is made of granitic rocks and the soil type is in the order of Spodosols (Tjia, 1988).

Fig. 2 : The location of study sites in Peninsular Malaysia

Plate 2 : Aerial photograph of the study site at Cameron Highlands


2



enlarged from the original scale of 1:63,630; figures in feet

Legend :

⋯⋯	Location of plots
~	Principal road
⌇	Rivers
x—x	Boundary between the states of Pahang and Perak

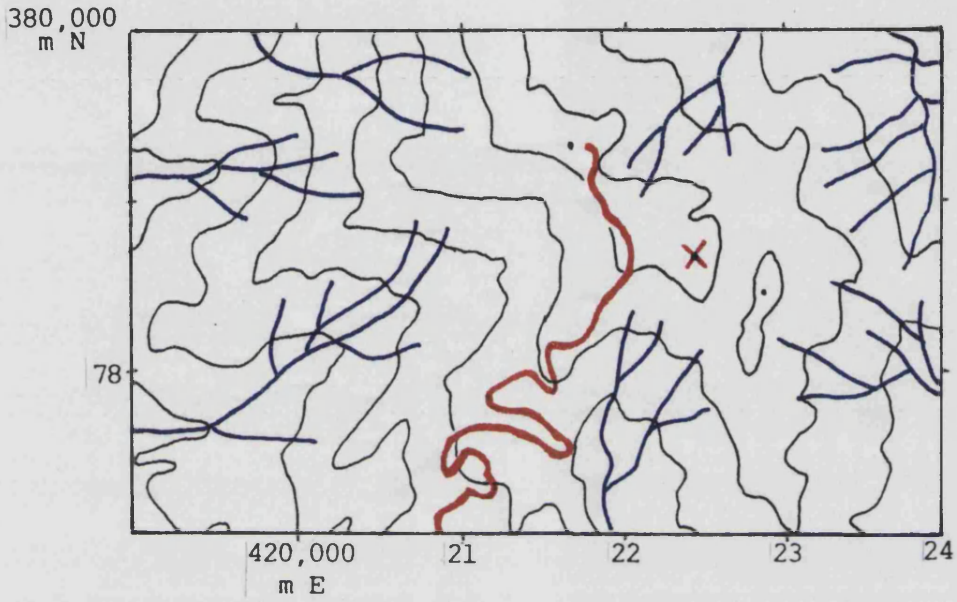
Fig. 3 : Topography of the study site at Cameron Highlands (CH)

The study site is in montane ericaceous forest. This forest formation, commonly referred to as upper montane or mossy forest is characterized by trees with gnarled stems and dense subcrowns with a luxuriance of bryophytes and pteridophytes. It is dominated by trees such as *Eugenia* sp., *Leptospermum* sp., *Rhododendron* sp. and understorey plants such as *Vaccinium* sp., *Pinanga* sp. and *Bambusa sp.* (personal observations) (Appendix 3).

3.2.1.1.2 Genting Highlands (Mt. Purun)

The second study site is located in Genting Highlands (Lat. 101° 47'E; Long. 3° 25'N) which is about 56 km away from Kuala Lumpur (Fig. 2). It is situated in the Bukit Tinggi Forest Reserve in the state of Pahang which forms part of the Titiwangsa Range. The terrain of the area is steep with an elevation ranging from 1696 to 2011 metres above sea level (Fig. 4). The site selection was based on similar reasons to those for Cameron Highlands. The area is drained by the Keshar, Berdah and Woh rivers. The mean monthly rainfall is 294.5 mm with a mean monthly air temperature of 19.7 °C and mean relative humidity of 53.7%. It is wet throughout the year (Fig. 5b) (Tan, Resort World Sdn. Bhd., pers. comm.). Much of the area is made of granitic rocks (Tjia, 1988).

The forest type is montane ericaceous, the dominant plants being *Rhododendron* sp., *Dacrydium* sp., *Calamus* sp., *Eugenia* sp., *Pandanus* sp., orchids, ferns and bryophytes (personal observations) (Appendix 4). This area is very disturbed by human. There is a long history of development and, even to date, portions of the surrounding forests are still being cleared or earmarked for hill resort development. The forest here is extremely fragmented and isolated.



enlarged from the original scale of 1:63,630; figures in feet

Legend :

- Location of plots
- Principal road
- Rivers

Fig. 4 : Topography of the study site at Genting Highlands (Mt. Purun)

Fig. 5a : The monthly changes in mean temperature and total rainfall in Cameron Highlands (January 1993-May 1995)

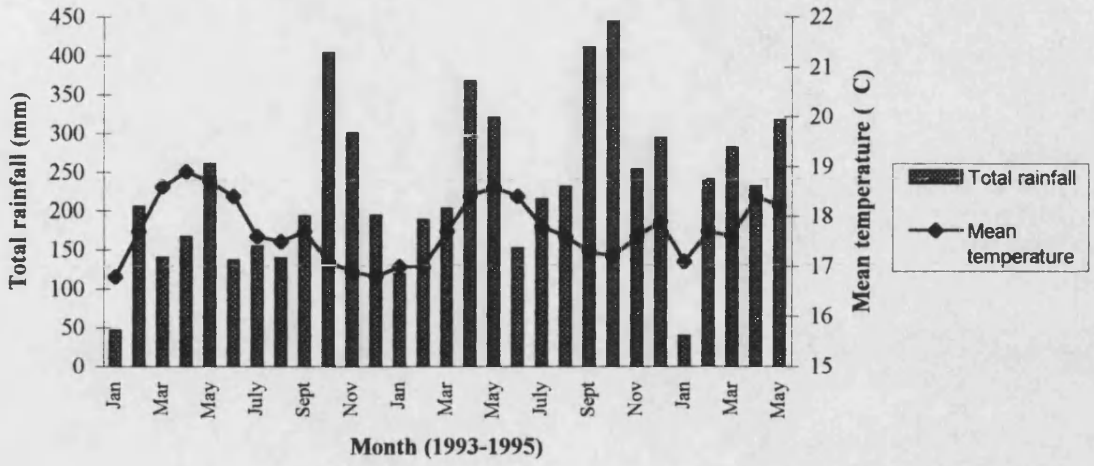
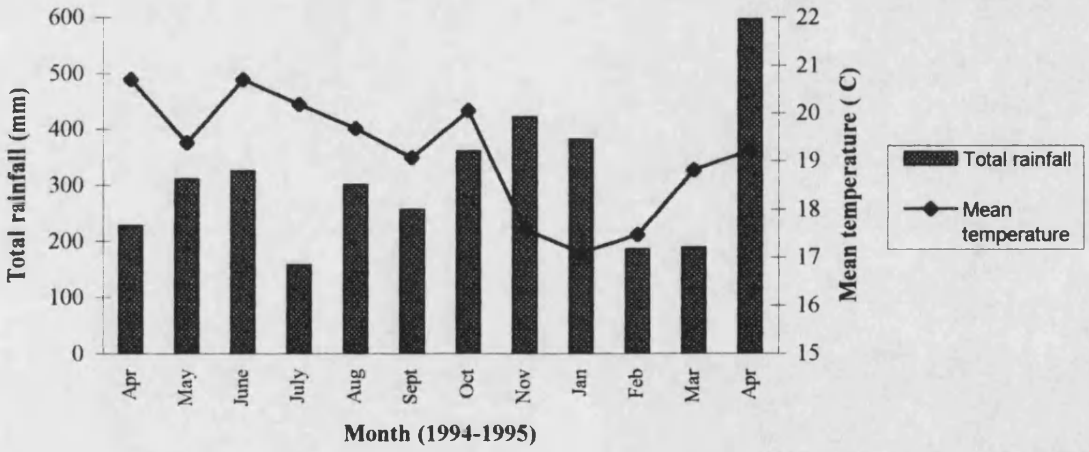


Fig. 5b : The monthly changes in mean temperature and total rainfall in Genting Highlands (April 1994-April 1995)



3.2.1.2 Sampling and plot establishment

3.2.1.2.1 Sampling

In Cameron Highlands, one hundred 10 x 10 metres plots, were marked out at regular intervals, alternating on both sides of the trail between Mt. Brinchang and Mt. Irau (Fig. 3). The trail was the baseline and it ran across a ridge, into a valley and a small plateau before reaching another ridge. The total ground area covered in this study is one hectare. A preliminary survey on the pattern of distribution was conducted. Consequently, sampling through systematic plot design was used (Greig-Smith, 1983). The location of each 10 x 10 metres plot was based on the preliminary survey and practical reasons.

In Genting Highlands, a non-randomized plot design was used. The site, which was demarcated on Mt. Purun (Fig. 4), was deliberately chosen so as to contain all the individuals selected for the reproductive study. Sixty-four 10 x 10 metres plots were marked out adjacent to each other, resulting in a Latin square of 8 x 8. The restriction to 0.64 hectare was a result of terrain difficulties.

3.2.1.2.2 Plot establishment

The plots were placed 10 metres apart, therefore the distance between the cornerpost in a plot to the corresponding post of the next plot is 20 metres. Distances between plots were measured using the tape measure and the existing trail as the baseline. To ensure that each side of the plot is perpendicular, a compass was used. Due to the presence of steep slopes and the variability in the angle of slope, it was necessary to offset the ground area in all plots. The 10 metres length of each side of the plot is accurate in the horizontal plane. The plot and subplot borders were demarcated by strings. Each plot was subdivided into four subplots of 5 x 5 metres each and each subplot was systematically given a number to prevent disorientation during mapping. This length was chosen because the area covered

enabled the individuals to be accurately mapped (Manokaran *et al.*, 1990). At least four people were required to demarcate a plot.

3.2.1.3 Plant enumeration and mapping

When the plot and subplots were established, enumeration of all individuals of *N. macfarlanei* began. Every plant occurring in the subplot was categorized into height classes, sex and habit. Six height classes were used *i.e.* less than 0.5 metres, between 0.5 and 0.99 metres, between 1 and 1.99 metres, between 2 and 2.99 metres, between 3 and 3.99 metres and more than 4 metres. Two categories of habit were used *i.e.* terrestrial and epiphytic. In cases where adult plants did not indicate their sex during the period of enumeration, they were tagged and observed regularly for flowering. Due to practical reasons, only plants of more than 4 metres high were tagged.

After each plant was classified and given an identification number, its position to the nearest X and Y borders was measured using a tape measure attached to a bamboo stick and mapped onto a graph data sheet. The graph data sheet is a reduced format copy of the plot with the subplots outlined. Any part of the trail that falls into the plot was also included in the map. Apart from the morphological and spatial enumeration, details such as the angle of slope, north-south bearings and the presence of other plant species were also recorded.

3.2.1.4 Measuring errors and techniques used to reduce the errors

Several kinds of measuring errors occurred in this study. They were measuring errors created during the demarcation of plot, sampling errors created during the enumeration of plants and mapping errors created during the measurement of the plant position.

During the demarcation of plot, the presence of a large tree may obstruct the reading of the compass. In addition, measuring error is incurred during the offsetting of slopes. If the plot was not carefully established, the steepness of the slopes will increase the total error rapidly. To reduce such errors, compass reading was staggered and measuring tapes were kept at uniform height and taut at all times. Endposts were kept vertical. Only experienced ecologists were used in the reading of compasses and the demarcation of border. All the hundred plots were demarcated within a single period of time.

Sampling and mapping errors arose during plant enumeration. The sampling error arose when plants occur in a cluster and when the searching pattern was not systematic. To reduce the first error, plants were examined thoroughly to ensure that they were separate individuals. This was done by looking closely at their root systems. To reduce the second error, the search for plants in all subplots followed a clockwise pattern.

The mapping error arised particularly when a plant is epiphytic. To reduce the error, the ground position of an epiphytic plant was determined in the following manner. A bamboo stick was dropped vertically down to the ground from the position of plant on the tree. From this point, the position to the nearest X and Y borders were measured. To further reduce the sampling error, the same group of enumerators were used throughout the study and plants were enumerated as soon as the plot was established.

Typographical errors were reduced by proof-reading the computer records against the original data sheets.

3.2.1.5 Statistical analysis

The data gathered were subjected to statistical analysis. The Poisson distribution approach was used to test the randomness of plant distribution in all plots (Sokal & Rohlf, 1981; Christie, pers. comm.). Two tests were used to measure the departure from randomness *i.e.* variance : mean ratio test (Blackman, 1942) and the chi-square test of goodness fit (Greig-Smith, 1983). These two tests were selected as they are the most common tests used in the analysis of plant distribution data. The softwares used for calculating these tests and mapping of plant distribution were Microsoft Excel version 5 developed by Microsoft Corporation, USA and Minitab release 6, developed by Minitab Inc.

3.2.2 Phytogeography

Collecting trips were conducted to selected peaks to determine the occurrence, phytogeographical limits and pattern of distribution of *N. macfarlanei* (Fig. 6). The peaks that were selected are listed in Table 2.

Table 2 : Location of selected peaks

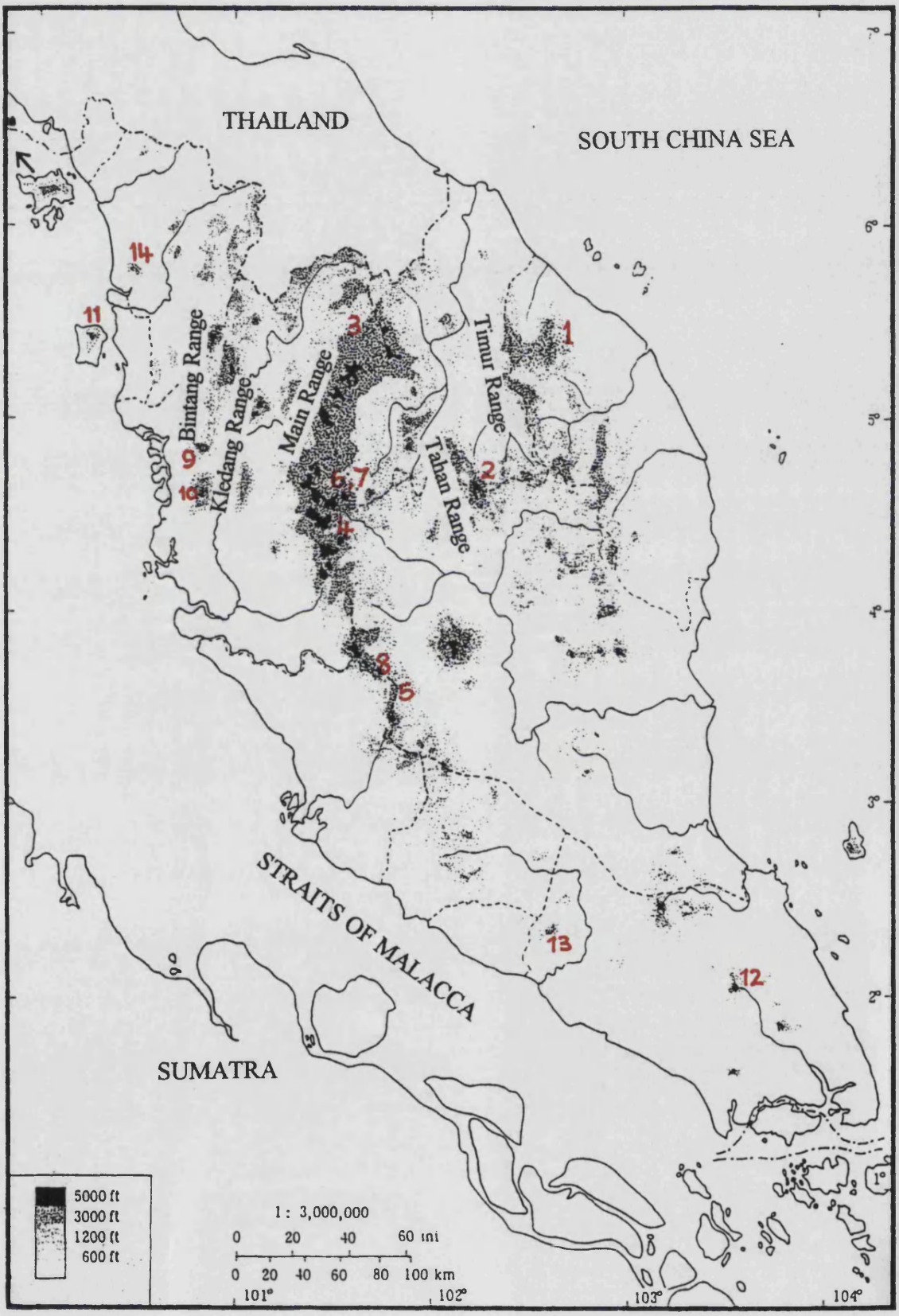
No.	Name of Peak	Mountain Range/Isolated Peak	State	Height (m asl)	Collection Dates
1	Mt. Lawit	Timur Range	Terengganu	1519	25-27/7/94
2	Mt. Tahan	Tahan Range	Pahang & Kelantan	2187	3-10/5/94
3	Mt. Kabut	Titiwangsa Range	Perak	1317	22-29/8/94
4	Mt. Batu Putih	Titiwangsa Range	Perak	2131	20-23/9/94
5	Mt. Rajah	Titiwangsa Range	Selangor & Pahang	1683	26-29/1/94
6	Mt. Jasar	Titiwangsa Range	Perak & Pahang	1981	3-4/10/93
7	Mt. Berembun	Titiwangsa Range	Perak & Pahang	1986	1-2/10/93
8	Fraser's Hill	Titiwangsa Range	Selangor & Pahang	1455	25-27/4/94
9	Mt. Hijau	Bintang Range	Perak	1448	6-8/9/94
10	Mt. Bubu	Bintang Range	Perak	1657	10-15/3/95
11	Penang Hill	Isolated Peak	Penang	730	6-7/1/95
12	Mt. Belumut	Isolated Peak	Johor	1010	28-30/10/93
13	Mt. Ophir	Isolated Peak	Johor	1276	3-4/6/93
14	Mt. Jerai	Isolated Peak	Kedah	1217	7-11/7/93

The selection of peaks was based on :

1. accessibility. With the exception of isolated peaks, the collection date listed for each peak was the actual number of days taken to reach the peak from the nearest habitation. This includes the time spent in doing the collection.
2. previous collection from the locality. Mt. Lawit, Mt. Kabut, Mt. Batu Putih, Mt. Rajah, Mt. Berembun, Mt. Hijau, Penang Hill, Mt. Jerai and Mt. Belumut have no previous record of collection. Collection records for the other peaks are old and this made it necessary to recollect the specimens for confirmation purposes.
3. permission granted by the Forestry Departments of respective states to enter and collect plants from a particular forest.

In cases where there were no flowers or fruits, materials were collected as voucher specimens. Materials with flowers or fruits were collected as herbarium specimens. Eight duplicates were collected and distributed to selected herbaria around the world. During collection, details such as pitcher, fruit and flower morphology, locality, habitat and habit were described. Herbarium specimens were prepared according to the protocol developed by the Royal Botanic Gardens, Kew, England (Bridson & Forman, 1992).

Fig. 6 : Location of selected peaks (see Table 2 for further details)



3.3 RESULTS

Analysis of the results shows that the spatial distribution of *N. macfarlanei* plants at both study plots was not random (contagious). The χ^2 values for the distributions at CH and Mt. Purun were 579.9 and 128.6 respectively; both values were highly significant ($P<0.001$) (Appendices 5 & 6). The variance:mean ratios for both plots were 32.19 and 10.18 respectively (Appendices 7 & 8). The significance of the observed χ^2s and the greater than 1 ratio values were primarily due to the occurrence of many sample plots without any individuals (Figs. 7 & 10).

The detection of non-randomness in plant distribution is influenced by two factors *i.e.* experimental errors and plot size (Skellam, 1952; Greig-Smith, 1957). In the case of *N. macfarlanei*, the experimental errors may include sampling and mapping errors. Skellam *loc. cit.* and Greig-Smith *loc. cit.* have demonstrated the effect of different plot sizes on the detection of non-randomness in plant distribution; this effect was also demonstrated during the sampling of *N. macfarlanei* population at Mt. Purun. As the plot size increased, the variance:mean ratio increased sharply, thus providing a scale at which non-randomness occurred. (Appendix 9). Although plot size has been shown to be an important criterion in the detection of non-randomness, Pielou (1961) suggested that the degree to which individuals are 'mingled together' is an intrinsic property of the population and is somewhat independent of scale. In the case of *N. macfarlanei* populations at CH and Mt. Purun, although the above-mentioned factors cannot be eliminated, the extremely large deviation from Poisson distribution in both tests could only have arisen from the intrinsic attribute of the populations.

3.3.1 Cameron Highlands

The total number of plants occurring in 1 hectare was 1667. The mean number of plants in a plot was 16.7 ± 23.17 and the density ranged from 0 to 109 plants. In many plots, the sample variance was larger than the sample mean, indicating that the general contagious distribution of the population is apparent even within a single plot (Appendix 7). 30% of the plots had no plants while only 1% had more than 100 plants (Fig. 7). 26% of the plots had less than 10 plants while 15% had more than 40 plants. The frequency of occurrence at densities between 1 to 36 plants remained fairly constant.

Fig. 8 illustrates the changes in the density of plants in 100 plots. The fluctuations in density were markedly related to the changes in the terrain of the study area. Most of the sampled plants occurred either on ridges or plateau. Plots on the plateau generally contained higher densities compared to those on ridges; 18% of the total density in the sample population occurred there. The plot with the highest density of plants was also recorded from the plateau (Appendix 10). Taking into account that only 3% of plots were there, the contribution to the total density is particularly high. Although most plots on the ridge zones had plants, there was a great deal of variation in the density of plants. This is in contrast to those placed at valley slopes where most plots did not have a single plant. The plants here contributed a mere 5.6% to the total density, of which a large proportion was contributed by slope-positioned plots close to the plateau. It is interesting to note that the density increased abruptly from zero as the slope-positioned plots got closer to the plateau (Fig. 8). Apart from the influence of the terrain, the vegetation of the area also affected the distribution. Plots predominantly occupied by bamboos and rattans did not have any plants.

Fig. 7 : Number of sample plots falling into various density classes at CH

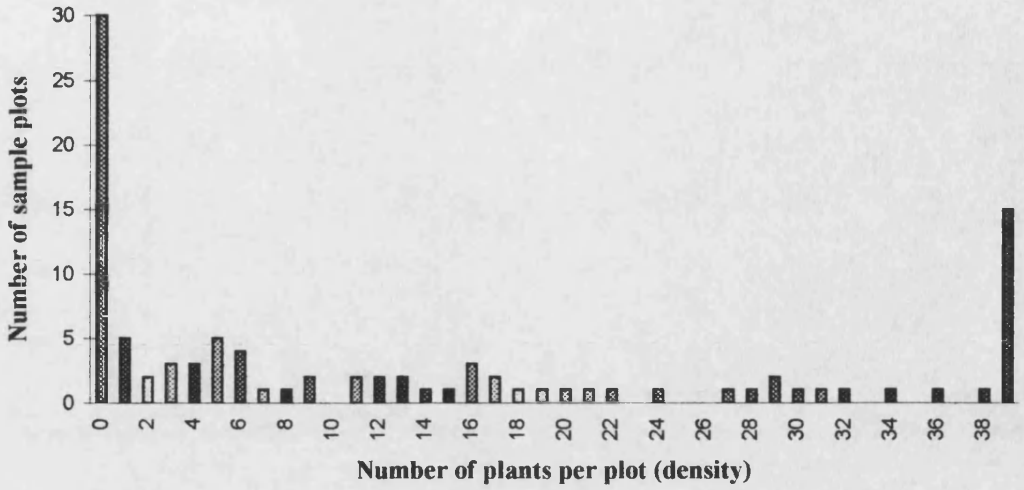
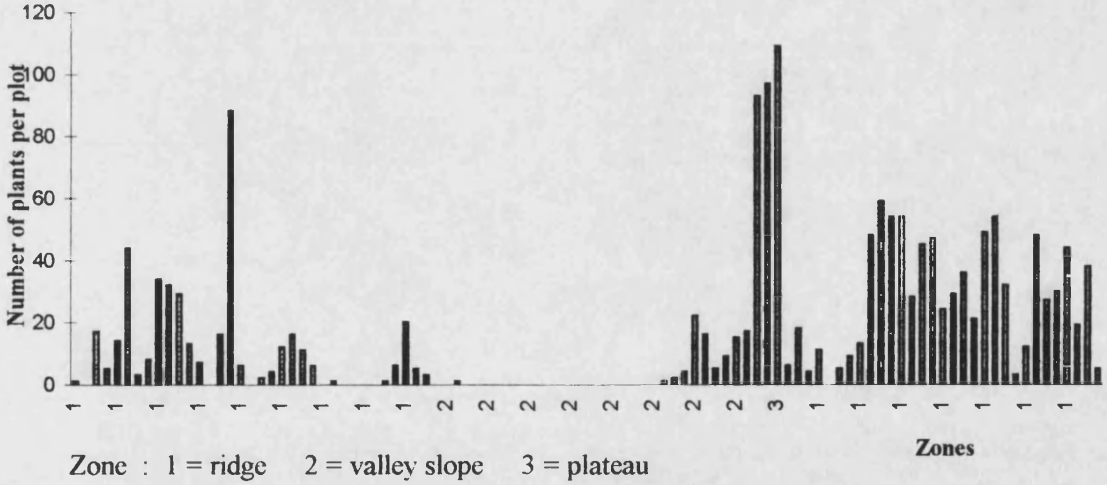


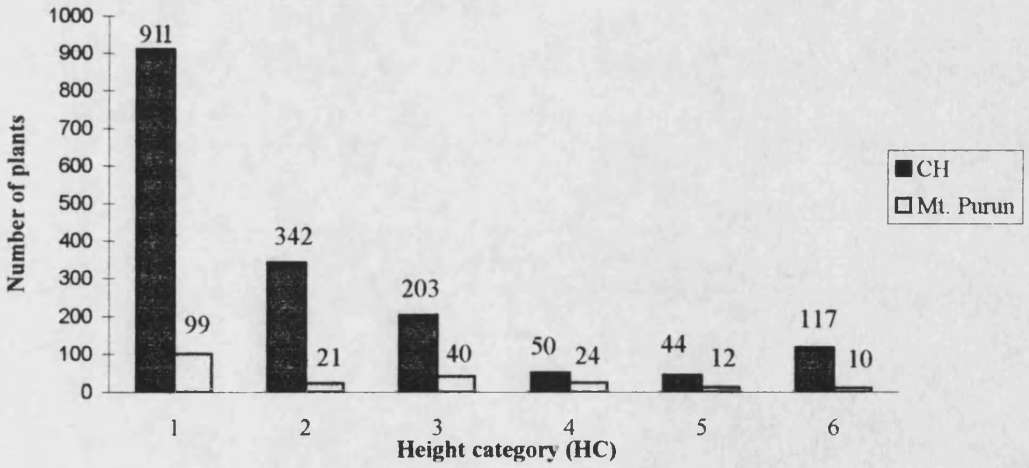
Fig. 8 : Histogram representing the density of *N. macfarlanei* in plots placed across three zones of mountain terrain at CH



Zone : 1 = ridge 2 = valley slope 3 = plateau

N. macfarlanei presented a left-skewed, height category frequency distribution in the 1 hectare plot. The frequency ratio was significantly different from 1:1:1:1:1:1 (χ^2 =1635.7, P<0.001). 54.7% of the sampled plants was in rosette stage (HC 1) (Fig. 9), followed by 20.5% in HC 2, 12.2% in HC 3, 3% in HC 4, 2.6% in HC 5 and 7% in HC 6. The highest frequency of plants in HC 1 suggests that active regeneration was taking place in the population at the time of sampling. Most of the rosette plants in the sample population occurred on the ridges and plateau; 39.7% occurred on the ridges while 13.3% occurred on the plateau (Appendix 11). The percentage of rosette plants occurring on the plateau is remarkably high considering that there were only three plots there. In these plots, 74% of the plants was in rosette stage and the remaining percentage was distributed between HCs 2, 3 and 4; there were no plants above 3 metres (HCs 5 & 6) here. All plants above 4 metres in height were confined to the ridges.

Fig. 9 : Number of plants in each height category in the sample populations at CH and Mt. Purun



HC : 1 = < 0.5 metres
2 = 0.5 - 0.99 metres
3 = 1.0 - 1.99 metres
4 = 2.0 - 2.99 metres
5 = 3.0 - 3.99 metres
6 = > 4.0 metres

The ratio of epiphytic plants to terrestrial plants in the sample population was almost 1:1 (Appendix 12a). This indicates that the probability of a seed landing on a stem and on the ground and the chances of successful establishment on either substrate are almost equal. This is not unexpected as wind-dispersed seeds occur at random and water is not a limiting factor in montane forests (Kapos & Tanner, 1985). Apart from reduced water stress (Grubb & Whitmore, 1966), the direct supply of liquid water droplets provided by the fog, assures seeds of sufficient amount of water for germination (Grubb, 1977). The ability to regenerate on many tree species also indicates that bark toxins do not affect establishment (Frei, 1973; Johansson, 1974) and that the species is specially adapted to conditions of low mineral nutrition (Smythies, 1963). Although the ratio in the sample population was almost 1:1, it is interesting to note that at the plateau, the ratio of terrestrial plants to epiphytic plants was 1.7:1 (Appendix 12b). The shift in the ratio might have been influenced by the heath vegetation and microclimate at the plateau. In general, the vegetation here has a less diversity of epiphytes compared to that at the ridges, possibly indicating that the microclimate may not be conducive for the growth of epiphytes and that there is a lack of suitable substrate, likely in the form of suspended soil or humus in the bark of trees (Wolf, 1994).

Only 1.5% of the total number of plants indicated their sex during the enumeration period (see 4.3.1 for Table 3). The ratio of male:female plants in the sample plots was 1:1. The number of male and female plants are believed to be an underestimate due to several factors. The 100 m^2 plots and their systematic placement along a transect would have missed out plants that flowered outside the plot borders. In addition, plants that had flowered much earlier on may have dropped their inflorescences/infructescences. Because the short period of enumeration could not provide an accurate picture, the period of observation was extended to 15 months (see 4.2.1 for further details).

3.3.2 Mt. Purun

The total number of plants occurring in the sample plots was 206. The mean number of plants in a plot was 3.2 ± 5.72; the density ranged from 0 to 24 plants (Appendix 8). The variance:mean ratio was greater than 1, indicating that the distribution is contagious. 59% of the plots had no plants while only 14% had more than 9 plants (Fig. 10). 27% of the plots had less than 9 plants. The total number of plants in the sample population is believed to be an underestimate because there were a few juvenile plants in the plots that cannot be identified to the species level and, as a result, they had been excluded from the enumeration and mapping exercise.

Fig.10 : Number of sample plots falling into various density classes at Mt. Purun

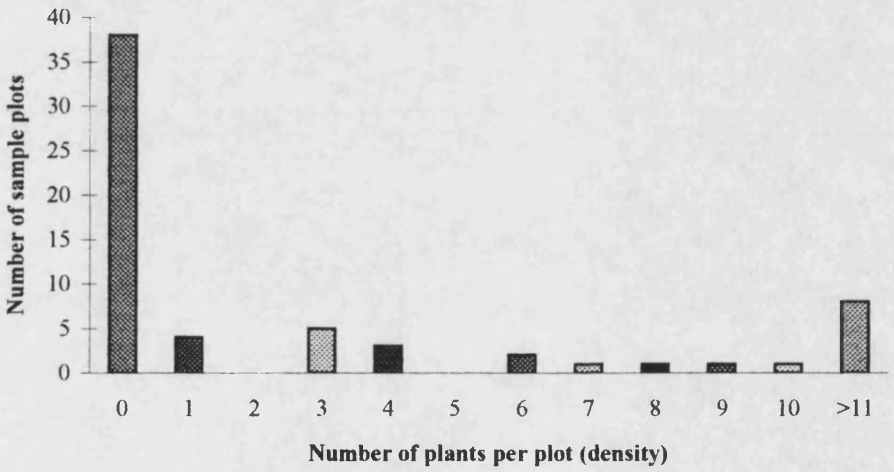


Fig. 11 illustrates the spatial distribution of plants in the sample plots. The distribution was markedly related to the micro-topography of the study site. The slightly off-centre cluster, which consisted 41.7% of the sample population (Appendix 8), occurred on a plateau of a small hill while the far bottom left and right clusters occurred on an exposed ledge of the hill. Plots facing west were practically empty of plants, probably because of the presence

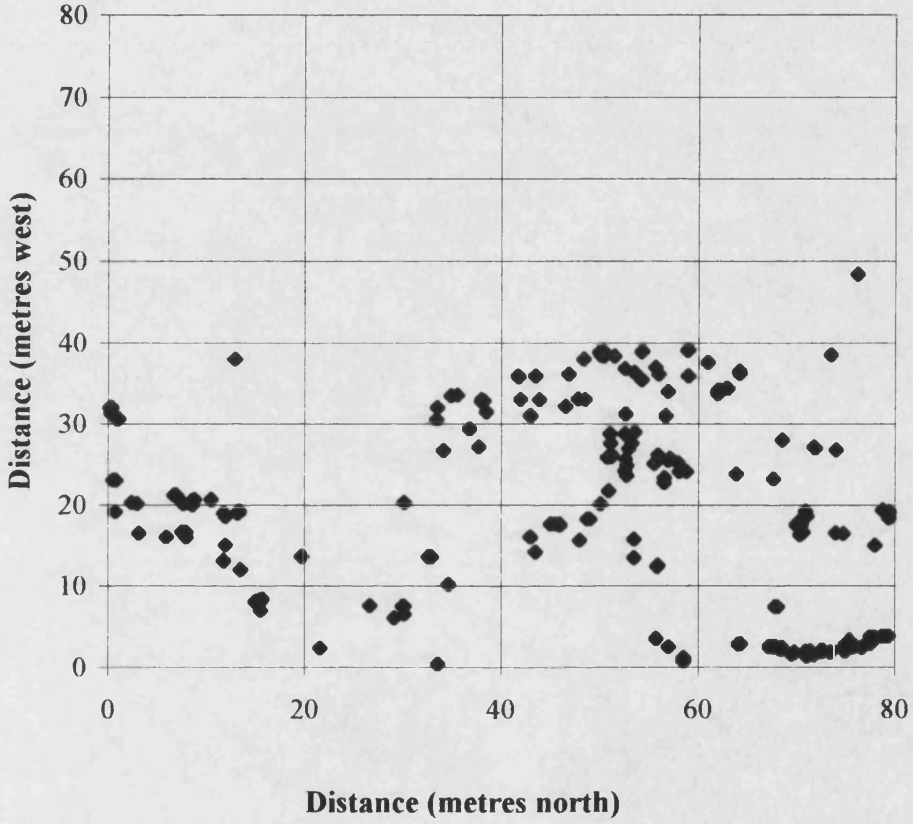
of large individuals of thorny *Pandanus* sp. In general, slopes facing north-east had more plants compared with slopes facing south-east (Fig. 11).

The frequency of plants in different height categories is strongly skewed to the left at the time of sampling (Fig. 9). The frequency ratio was significantly different from 1:1:1:1:1:1 (χ^2 = 133.35, P<0.001), a result which was similar to that at CH. 48% of the plants was in rosette stage, followed by 10.2% in HC 2, 19.4% in HC 3, 11.7% in HC 4, 5.8% in HC 5 and 4.8% in HC 6.

The ratio of epiphytic plants to terrestrial plants is 1.4:1 (Appendix 12a). This ratio is slightly larger than that at CH, but it nevertheless indicates that a seed landing either on a stem or on the ground has equal opportunities to regenerate.

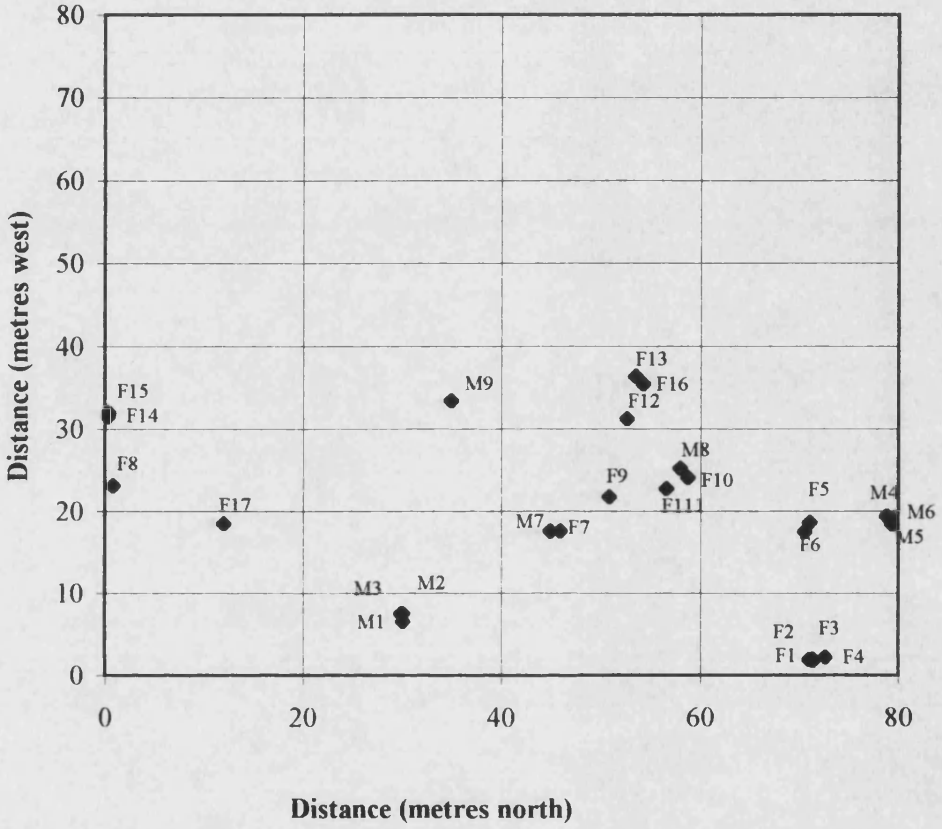
8.2% and 5.3% of the sample population were female and male plants respectively (Appendix 15); this gave a ratio of 1.5:1 female to male plants, a ratio which was not significantly different from 1:1 (χ^2 = 1.29; P>0.05). Analysis of the results shows that the spatial distribution of male and female plants was contagious (χ^2 =18.94; P<0.001) (Appendix 13) and this is in accordance with the result obtained for the spatial distribution of the sample population. The spatial distribution of male and female plants is illustrated in Fig. 12. The mean distance of the nearest male neighbour was 15.1 ± 11.02 metres (Appendix 23). 17.6% of the nearest male neighbour was further away from the lower limit (4.1 metres) while another 17.6% was further away from the upper limit (26.1 metres). The remaining 40% fell within the range. The nearest male distance ranged from 1 to 34.7 metres (see 4.4.3 for discussion). Figs. 11 and 12 clearly show that there was strong tendency for juveniles to occur around parents, indicating that factors related to

Fig. 11 : The spatial distribution of *N. macfarlanei* plants in the sample plots at Mt. Purun *



* total sample area is 0.64 hectares; each grid represents a plot

Fig. 12 : The spatial distribution of male and female plants in the sample plot at Mt. Purun



* total sample area is 0.64 hectares; each grid represents a plot
F = female
M = male

seed dispersal play a role in determining the extensiveness of a population (Cordova, 1979).

3.3.3 Phytogeography

N. macfarlanei is found in elevations of above 1450 metres asl and it is entirely associated with the upper montane forest characterized by the abundance of non-vascular epiphytes, canopy height of less than 18m (Whitmore, 1990) and a high incidence of low level cloud and fog (Grubb & Whitmore, 1966). Although individuals can sometimes be found at the elevation of 1190 metres asl in the lower montane zone which is characterized by canopy height of up to 33 m and reduced abundance of non-vascular epiphytes, these are few in comparison.

The species is found in four mountain ranges in Peninsular Malaysia but it is not known to occur in the eastern-most range *i.e.* Timur Range (Fig. 6). A recent survey to Mt. Lawit (Timur Range) did not indicate any populations, but this does not rule out the possibility that the species does exist there. As shown in the past, this could merely be the consequence of the lack of accessibility, resulting in less comprehensive botanical surveys. All peaks surveyed from the southern and central parts of the Titiwangsa Range contained populations of *N. macfarlanei* and the distribution is most extensive in these regions. This is also supported by the extensive collections of previous botanists. The most northern locality where the species is known to occur is at Mt. Korbu and there has been no past citation of the species occurring further up north. No population was recorded from Mt. Kabut but this could merely be due to the lower elevation. This does not imply that Mt. Korbu is necessarily the northern limit of its phytogeography.

To date, there has been no record of the species occurring on isolated peaks. None of the five surveyed peaks scattered in Peninsular Malaysia appeared to have any populations. As there has been no previous record, it was not likely that the populations had died out due to adverse conditions experienced at those localities. In addition, Mt. Hijau which is situated close to two ranges, did not have the species. The nearest peak to it, Mt. Bubu, is hardly 50 kilometres away but had large numbers of plants. One may suggest that demographic and environmental factors existing on Mt. Hijau essentially limit the establishment and growth of this species. However, several studies conducted on outlying mountains such as Mt. Belumut in Malaya (Holttum, 1924), Mt. Dulit (Richards, 1936) and Mt. Silam in Borneo (Proctor *et al.*, 1988; Bruijnzeel *et al.*, 1993), Mt. Payung in Java (Hommel, 1987), the Louisiades in New Guinea (Brass, 1959) and peaks in Krakatau islands (Whittaker *et al.*, 1989) have shown that isolated mountains, although being much lower in elevation, have similar vegetation types, physiognomy and climatic conditions to those on adjacent large massifs. This effect, referred to as the *Massenerhebung* effect, was also observed in many of the isolated peaks visited (the exception is the Penang Hill) (Chua, personal observations). A more plausible explanation is the lack of arrival of the species in the neighbouring peaks, which reflects restrained seed-dispersal distances. The dispersal mechanism is likely to influence the ability of the species to occupy new localities. Although no *N. macfarlanei* occurred on those peaks, species such as *N. albo-marginata*, *N. sanguinea* and *N. ampullaria* were frequently found.

The earliest collection of *N. macfarlanei* from Peninsular Malaysia was in 1885, unfortunately no locality was recorded (Appendix 2). The second collection was made in 1877 from Mt. Bubu in Perak. Between that year and 1900, four more collections were made, three from Mt. Bubu and one from Mt. Batu Putih in the state of Pahang. Recent assessment at these peaks showed that after more than a hundred years, the populations are

still thriving and viability, as reflected from the density of juveniles, is stable. Although not all of the peaks surveyed by previous botanists were selected for reassessment, those that were selected had thriving populations. Since then, some of these peaks had experienced some degree for habitat fragmentation as a result of development activities. It is reassuring to note that despite the fragmentations, the populations have not become extinct. Unfortunately, the extent of changes in the population size and the loss or gain in genetic diversity, that occurred between those years, cannot be assessed and the influence of forest fragmentation on viability cannot be gauged.

3.4 DISCUSSION

3.4.1 Phytogeography

N. macfarlanei is restricted to elevations above 1450 m in the mountainous ranges of Peninsular Malaysia. The information available at the present time indicates that the species occurs most extensively at the central and southern parts of the Titiwangsa Range, with the southern limit of the species corresponding with the southern end of the range. The northern limit is at present unknown because of the paucity of collection arising from the lack of accessibility. In relation, it is not known how the seasonal climate, as experienced by vegetation in most habitats at the northern part of the country, influences the phytogeography of the species. Its distribution extends to the Tahan Range in the East and to the Bintang Range in the West. Both these ranges are less extensive in size compared to the Titiwangsa Range although the upper montane altitudinal zones are comparable. It would therefore be not unexpected to find fewer populations in these ranges because of the reduction in the number of suitable habitats. The populations recorded by previous botanists on selected mountain peaks have remained intact, indicating that despite forest fragmentation, populations have not actually gone extinct. Nevertheless, some of the genetic diversity may have been lost as a result of forest fragmentation, but this might be difficult to detect because of regular recombination of genes resulting from its allogamous breeding system (see 4.4.2 for further details).

The species does not occur on certain isolated peaks even though these may be in close proximity with peaks containing populations. The ability, therefore, to colonize new peaks would appear to be hampered by the plants' seed dispersal mechanism. Wind dispersal occurs at random and many studies with a range of tropical species have shown that regeneration of seedlings often occurs close to parent plants (Bawa & Crisp, 1980) (see 4.3.2.2 for arguments related to the dispersal mechanism). Although wind speed at higher

elevation is stronger compared to the lowlands (Shreve, 1914; Gleason & Cook, 1927; Baynton, 1968; Lawton, 1982), and is likely to extend the distance of seed travel, vegetational barriers provided by the canopy of leaves and branches will probably reduce this distance. The lack of colonization between peaks which are less than 50 kilometres apart clearly indicates that the effective seed travel is less than this distance. Within the observed population at CH, the sudden increase in the density of plants as the distances to parents are reduced (Fig. 8) is again a reflection of the mechanics of wind-dispersed seeds. In addition, the marked absence of individuals at the slopes (Fig. 8), which lie between the ridge and plateau, apart from being the result of the physiological and environmental constraints that act on individuals which manage to get established there, possibly indicates that the in-coming number of seeds to the slopes, dispersed by the established populations on the ridge and plateau, is relatively low. Although persistent strong winds are common in upper montane forests (Shreve, 1914; Gleason & Cook, 1927; Baynton, 1968; Lawton, 1982), slopes have generally been shown to be more sheltered from wind movements, thus reducing the arrival of seeds into the area (Schuster, 1957; Benzing, 1981). New populations will therefore be confined to the vicinity of the parent populations and long-distance geneflow *e.g.* between mountain peaks, is unlikely to occur naturally. This isolation, which is presumably due primarily to the restricted movement of propagules, does not imply that the species has no physiological capacity to colonize new peaks.

N. macfarlanei is clearly a habitat specialist and, as a result, it is a classic example of an environmental endemic that is restricted in its distribution by specific climatic and edaphic processes (Kruckeberg & Rabinowitz, 1985). The sparse occurrence in the lower montane forest formations indicates that conditions there are somewhat unfavourable to regeneration and growth. The similarities in the montane environment between the

mountain ranges of Peninsular Malaysia may eventually result in a greater degree of distribution continuity, particularly if human intervention occurs.

3.4.2 Spatial distribution of populations

There seemed to be a great deal of similarity between the spatial distribution pattern of *N. macfarlanei* at the two study sites although the distance between them is almost 200 kilometres. Within a mountain range, this species seemed to have an affinity for ridges and plateaus but not for slopes (Fig. 8). This affinity was also observed during documentation trips at selected peaks around the peninsular. Stochastic factors particularly those relating to environment such as soils, topography and climate are strongly believed to influence the spatial distribution of plant populations (Kruckeberg & Rabinowitz, 1985); the fact that populations of *N. macfarlanei* occur in two distinctly different microhabitats (ridges and plateaus) indicates that the species possesses a certain degree of physiological plasticity. The microclimate at the plateau differs from that at the ridges and is more comparable to those at forest edges (Marquis, 1965; Wales, 1967; Lovejoy *et al.*, 1984; Kapos, 1989; Matlack, 1993) and gap formations in lowland canopied forests (Schulz, 1960; Fletcher *et al.*, 1985; Raich, 1987; Becker *et al.*, 1988). On the other hand, the microclimate at the ridges, although less extreme in intensities, is much more complex due to the variation arising from the filtering-off by several layers of canopy stratum (Sasaki, 1983). The possession of physiological plasticity and tolerance towards extremities in the environment in *N. macfarlanei* is made apparent by the presence of a high density of juveniles on the plateau and ridges. However, the results of this study also showed that the species avoids sheltered slopes and that this occurs in both the terrestrial and epiphytic life-forms. Although genetic and demographic stochasticities and natural catastrophes have been known to fragment plant populations (Shaffer, 1981), it is unlikely that these factors were the causes leading to a distinct fragmentation in the observed *N. macfarlanei* populations at

CH. Although no study was conducted to determine the factors influencing the spatial distribution of this species at this site, it is speculated that the lack of a suitable substrate for the germination and establishment of seeds and the light conditions at the slopes play crucial roles.

The first speculation is based on casual observations of the composition of tree species in the study site at CH showing that although tree diversity at the slopes did not differ from that at the ridges and plateau (Appendix 3), the growth habits of the species did differ between the microhabitats. Many of the trees on the ridges or close to the ridges have gnarled stems; they are densely covered with mosses, ferns and herbaceous flora and these provide an excellent substrate for the germination and establishment of *N. macfarlanei* seedlings. In contrast, many trees at the slopes, particularly those further away from the ridge, have straighter and thinner trunks and they do not support anything like the same profusion of epiphytes. The lack of a suitable substrate could perhaps explain why epiphytic *N. macfarlanei* are not found there. Such a selection was also reflected at the Mt. Purun site; here, the species do not occur in areas covered with *Pandanus* spp. and plants from the order of Bambusoidae.

The second speculation arises from the many ecological studies conducted in the lowland tropical forests which have shown that the structure and layering of the canopy influence to a great extent the light conditions of a particular area, giving rise to a complex array of local attributes. In particular, radiation has been shown to decrease with the increase in canopy level (Sasaki, 1983; ter Steege & Cornelissen, 1989) and tree height (Wolf, 1994). As a result, greater light variation is expected to exist on valley slopes, although this does not imply a change of other elevation-related climate attributes. *N. macfarlanei* seeds are

dependent on light for germination and this requirement may not be sufficiently provided for by the micro-environment on the slopes.

The high density of plants on the plateau site corresponds with the results of an unpublished work on the effects of different light quantities on the growth of *N. rafflesiana* where the germination percentage and dry biomass of leaves, stem and roots were much higher in treatments with higher light quantities. This finding possibly explains why *Nepenthes* spp. in general seem to favour exposed and degraded sites (Ashton, 1971; Shivas, 1984; Phillips & Lamb, 1988; Adam *et al.*, 1989); in line with these observations, Holttum (1940a), Chai & Radcliffe (1984) and Hotta & Tamin (1986) mentioned the lack of persistence in shaded areas. In contrast, however, the results of this study indicate that *N. macfarlanei* also occurs in more closed forests and this is in agreement with the observations of Smythies (1963) and Adam *et al.* (1992) on the distribution of certain other *Nepenthes* spp.

The relative abundance of juveniles at both study plots indicates that the species has a high regeneration capacity (Fig. 9). Results obtained from reproductive observations and germination studies showed that seeds are produced in large quantities and germination percentages often exceed 80%, given a suitable environment (see 4.3.4 and 5.3.3 for further details). The lower numbers of more mature plants (Fig. 9) is not unexpected as many species experience some form of regulation in their seedling populations (Martínez-Ramos,1991; Martínez-Ramos & Soto-Castro, 1993). Several factors may contribute to the 'self-thinning' effect at the juvenile stage (Yoda *et al.*, 1963; White & Harper, 1970). First, competition among juveniles will only select plants that are vigorous. In relation to this, the heterogeneous genotypes in seeds of *N. macfarlanei*, arising from the allogamous breeding system, will probably result in a large variation in the degree of fitness between seedlings. Second, although differences between the sizes and weights of mature seeds are

negligible between different fruits and parents (see 5.3.1), the different maturation stages of the ovules in a single flower (see 4.3.2.1 for further details) during the period of receptivity indicate that maternal investment probably varied in amount between ovules and, presumably, embryos. As a result, embryos that develop earlier are likely to be more fit compared to those that develop later (Lloyd, 1980). The mortality of the plants could therefore be the direct or indirect consequence of the level of maternal investment in the developing seeds (Werner, 1975; Solbrig *et al.*, 1980; Gross, 1981; Parker, 1982). Third, results from 5.3.3 indicate a marked staggering of germination times. Although no attempt was made in this study to germinate the seeds developed from a single parent, past studies have shown a strong correlation between seeds that germinate early and establishment success (Cook, 1980).

Overall, the almost equal ratio of epiphytic plants and terrestrial plants in both study sites would suggest that water is not a limiting resource in the montane forests. However, the habit is determined primarily by the vegetation and environmental conditions within a particular microhabitat; at CH, for example, the number of terrestrial *N. macfarlanei* at the plateau was almost twice the number of epiphytes (Appendix 12b). The heath vegetation at the plateau consists of trees of low height with densely branched trunks and, in general, plants grow close to each other, giving rise to a compact structure. This structure, together with the presence of a relatively extreme microclimate, do not support many epiphytes; it is therefore, not surprising that few *N. macfarlanei* plants get established as epiphytes here. The ability of this species to develop different habits may therefore, be regarded as an additional advantage, supplementary to its physiological plasticity for then not only are they able to occur in greater numbers in a given area but they are also able to occupy a wider range of microhabitats. The presence of two habits in *N. macfarlanei* may

partially indicate an opportunistic behaviour, a behaviour that is likely to be of considerable importance in determining whether a population is able to survive in altered habitats.

3.5 CONCLUSIONS

N. macfarlanei occurs most extensively in the southern and central parts of the Titiwangsa Range but it is does not occurs in all peaks. In addition, its population is discontinuous in the locality that they occupy; this discontinuity is largely influenced by a complex interaction of local topography, microclimate and seed dispersal ability. Although the species possesses some level of physiological plasticity with a relatively large tolerance range and may, as a result, be tolerant to a certain degree of habitat fragmentation, it will only select the optimal microhabitats for growth and development. Where suitable microhabitats occur, the regeneration capacity of the species is often relatively high. Although the results of this study showed that it would be correct to suggest that only the ridges and plateaus where *N. macfarlanei* occurs should be considered as target areas for conservation, it would be naive to implement such a recommendation since the loss of forests at the slopes would inevitably degrade the surrounding environment and thus negatively affect the species' ability to sustain its populations elsewhere.

CHAPTER 4

THE REPRODUCTIVE BIOLOGY OF *N. macfarlanei*

THE REPRODUCTIVE BIOLOGY OF *N. macfarlanei*

4.1 INTRODUCTION

4.1.1 The aim

The dynamics and viability of a species population are irrevocably linked to its reproductive capacity. The cumulative viability of populations across many habitats and localities in turn determines the conservation status of a species. Understanding the reproductive and breeding mechanisms of the species of interest has become a necessary tool in the attempt to determine the population dynamics, an approach which is frequently being used to indicate its status in the natural habitat (Gottlieb, 1973; Burgmann *et al.*, 1988; Karron *et al.*, 1988)

Models of population dynamics have attempted to derive figures for minimum population sizes required for the conservation of targeted species (Shaffer, 1987; Menges, 1990). These models are technically useful but to date they have had little practical application in the tropics because of the myriad of biological and man-imposed complications. It is a well-known fact that each region is peculiar in its floristic composition and within one region, countries have their own unique flora. As a result, successful conservation programmes in one country often do not apply to another country or region. In addition, as mentioned earlier, many resource managers or conservators of biological diversity lack a sound biological training which ultimately affects their ability to manage a reserve effectively. Sophisticated technology such as the molecular approach now used to determine the genetic diversity of a population is usually lacking in the less developed countries. Very often, the only tool available to such managers is simple field observation on the phenotypic changes occurring in the populations.

The information hitherto available on the reproductive biology of *Nepenthes* sp. has been very basic and some of it has not been entirely helpful for conservation. The extent of the present knowledge still leaves a huge gap. The behaviour and interaction of a particular species with other species in the context of communities have barely been described in either qualitative or quantitative terms. This study attempts to provide a qualitative description of the reproductive biology of *N. macfarlanei*, the possible interactions and implications for conservation.

4.1.2 Literature review

4.1.2.1 Reproductive morphology

Nepenthes spp. are dioecious whereby the male and the female flowers are borne on different plants. The inflorescence is borne terminally but later becomes displaced as the apical shoot further develops (Danser, 1928; Kurata, 1976). In climbing individuals, it often protrudes above the canopy. The flowers of *Nepenthes* spp. are arranged in either a raceme or a panicle. In species with a raceme inflorescence such as *N. macfarlanei*, *N. lowii*, *N. villosa*, *N. sanguinea* and *N. gracillima*, each pedicel bears one flower and it is subtended by a bract (MacFarlane, 1908; Kaul, 1982) (Plates 3c & 3d). In species with a panicle inflorescence such as *N. ampullaria* and *N. paniculata*, the branches are corymbous and each bears up to 10 flowers (MacFarlane, 1908; Danser, 1928). Although it might not have any phylogenetic significance, it is worthwhile noting that all the species endemic to the montane habitats in Peninsular Malaysia (*i.e. N. macfarlanei*, *N. sanguinea* and *N. gracillima*) have raceme inflorescences. Most species have ferruginous hairs on the rachis, peduncle and pedicels. In most species of *Nepenthes*, the male inflorescence is longer than the female inflorescence, and this is due primarily to the elongation of the rachis as the flowers mature acropetally. Danser (1928) recorded that the length of the male rachis of *N. macfarlanei* is between 9 to 25 cm long but at that time nothing was known of the length

Plates 3a - e : Development of the inflorescence and infructescence in *N. macfarlanei*

Plate 3a : Female inflorescence after exposure from leaf sheaths

Plate 3b : Male inflorescence after exposure from leaf sheaths

Plate 3c : Female inflorescence bearing receptive flowers

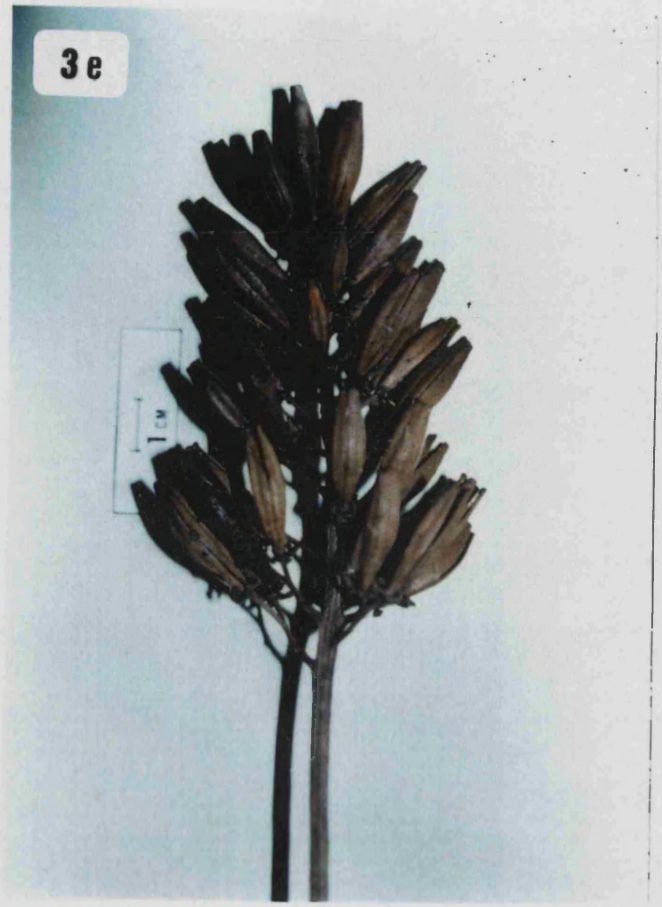
Plate 3d : Male inflorescence bearing flower buds


3 a


3 b


3 ç


3 d


3e
1 cm


4
JEOL/SEMAFORE/SYNOPTICS
FRIM

of the female axes. Recent studies conducted by Kato (1993) on *N. gracilis* showed that a male inflorescence had an average of 90 flowers while a female inflorescence had an average of 52 flowers.

The flower buds are globose and covered with tepals (Plates 3c & 3d). When the male flower opens, the androphore elongates to hold the anthers well above the tepals. The tepals, which may be free or overlapping, are rounded-elliptical (*e.g. N. macfarlanei*) or lanceolate and have numerous elliptically-shaped nectar glands on the inner surface. The latter are green, upwardly inclined and they exude copious nectar throughout anthesis, later becoming reflexed and brown after the pollen is shed (Kaul, 1982). Tepals of both male and female flowers behave similarly. In *N. gracilis*, nectar production starts in the evening and ceases in the morning and the sepals of male flowers produce more nectar compared to those of female flowers (Kato, 1993).

The anthers are sessile and are placed either in one whorl, with or without an apical group (as in *N. macfarlanei*) or in almost two whorls (Danser, 1928) (Plate 4). They are bisporangiate in *N. gracilis* (Lim & Prakash, 1973) and *N. lowii* (Kaul, 1982) while in *N. ampullaria* (Kuhl, 1933) and *N. melamphora* (Stern, 1917), they are tetrasporangiate. The anther opens extrorsely with longitudinal slits to release pollen grains. In *N. gracilis*, it was observed to dehisce between 4 and 6 p.m. (Lim & Prakash, 1973). The pollen grains adhere firmly to each other in tetrahedral tetrads (Lim & Prakash, 1973; Kaul, 1982) and they are three-celled in *N. lowii* and *N. villosa*. The outer surface (exine) of the pollen grain in *Nepenthes alata, N. pyllamphora, N. vieillardii, N. lowii, N. villosa* and *N. gracilis* is spinuliferous (Erdtman, 1972; Kaul, 1982; Kato, 1993). In *N. alata*, the diameter of the tetrad is 31 μ while in *N. phyllamphora* and *N. vieillardii*, they are both 27 μ (Erdtman, 1972).

The female inflorescence differs only slightly from the male, although it is more robust. In *N. macfarlanei*, the stigma is lobed and sessile while the ovary is somewhat angular with four locules, a central placenta and numerous anatrophic ovules (Danser, 1928). Kaul (1982) noted that *N. lowii* had an average of 307 ovules per flower. Embryological work on *N. gracilis, N. villosa* and *N. lowii* indicates that the ovules of *Nepenthes* spp. are crassinucellate with two layers of integument. After pollination, the outer integument continues to elongate around the embryo, eventually forming the filiform wings of the seed. The inner integument becomes papery, enveloping the embryo. In *N. gracilis*, the embryo develops transversely up to the filamentous proembryo stage, after which division at other planes takes place to produce a globular embryo, which soon differentiates into two cotyledons at the distal end to form the heart-shaped embryo (Lim & Prakash, 1973). At maturity, the embryo has well-developed, unequal cotyledons and a hypocotyl but it does not have a plumule. Although the Nepenthaceae have been reported as having endosperm in their seeds, there were none in *N. lowii* and *N. villosa* (Kaul, 1982).

The almost woody fruit of *Nepenthes* spp. is a fusiform, loculicide capsule and it is about 10 times larger than the gynoecium at maturity (Plate 3e). The seed, which is covered with a dense indumentum composed of stellate hairs, is light and according to Danser (1928) it is dispersed by wind. Kaul (1982) noticed that only a few seeds in *N. lowii* contained mature embryos; in the twenty four fruits that he selected, each had an average of 176 seeds, but only an average of eight seeds contained embryos. Although the other seeds were of the same size as the fertile ones, they had no embryos. This is in contrast with the Phillipps & Lamb (1988) observation that between 100-500 seeds may be found in a capsule of an unspecified *Nepenthes* sp. and Danser's (1928) observation that a higher number of fully-developed seeds can be found in capsules located primarily at the proximal end of the infructescence.

Most of the present taxonomic information was derived from Harms' (1936) revision of Danser's (1928) account. Although both accounts have provided much needed information, many of the uncertainties in the family's life histories could not be resolved due to the lack of proper herbarium collections. Since that time, more collections have been made from different habitats and it is hoped that many of the taxonomic uncertainties mentioned in both of their accounts can be sorted out. The family is presently being revised under the Flora Malesiana project by a team of taxonomists from the Royal Botanic Gardens, Kew and Trinity College Dublin, Ireland (Jebb & Cheek, pers. comm.).

4.1.2.2 Flowering and flower visitors

Although Holttum (1940a) suggested that the flowering of *Nepenthes* spp. is seasonal, he did not explain how he reached this conclusion. Very little is known about the pollination mechanism and syndrome in *Nepenthes* spp. The flowers of *Nepenthes* spp. would seem to have the potential for attracting insects because of their nectar secretion and foetid odour (Lim & Prakash, 1973; Kaul, 1982). Daumann (1930) and Kato (1993) have demonstrated the presence of sugars in the floral nectar of *N. mixta* and *N. gracilis* respectively, thereby confirming that there is food available for foraging invertebrates. It was hardly surprising therefore that Adam *et al.* (1989), in their study on five Bornean species *(N. villosa, N. kinabaluensis, N rajah, N. reinwardtiana* and *N.* aff *fusca*), reported insects as the commonly encountered visitors. Diptera were found to be the most frequently encountered visitors on *N. rajah* (65-71% occurrence), *N. villosa* (83-94%) and *N. kinabaluensis* (90-100%) while Hymenoptera were the more frequently encountered visitors on *N. reinwardtiana* and *N.* aff *fusca;* other groups of visitors were Coleoptera, Lepidoptera, Hymenoptera and Formicidae. Unfortunately they did not attempt to identify the flower visitors below the family level and they made no speculation as to potential groups of pollinators. Kato (1993), in his study with *N. gracilis*, reported that flies (Diptera) and

moths (Lepidoptera) were the commonly encountered visitors; most of the visitation took place between early evening and midnight and the frequency of visits varied significantly between days.

4.2 MATERIALS AND METHODS

4.2.1 Phenology

Plots in Genting Highlands and Cameron Highlands were used in this study. The site in Cameron Highlands was used for monthly flowering/fruiting phenology observations while the site in Genting Highlands was used for weekly monitoring of flower & fruit development, floral biology, pollination and pollinator studies.

Phenological observations and experiments at Genting Highlands began in August 1993 and were terminated in July 1995. Phenological observations at Cameron Highlands began in September 1993 and were terminated in December 1994. The mean temperature and rainfall pattern in both sites during the duration of observation are shown in Figs. 5a & 5b. The meteorological data from Genting Highlands is unpublished and was obtained from Resort World Sdn. Bhd., a public-listed company while the data from Cameron Highlands was obtained from the Meteorological Department.

4.2.2 Flower and fruit development

During the population study at Genting Highlands, all reproductive individuals in the plot were tagged and they were constantly inspected for fresh signs of flowering. Inflorescence and flower initiation take place within several leaf sheaths and there are no external morphological changes in the plant to indicate that the flowering has been initiated. At a more advanced stage however, the sheaths bulge conspicuously, enclosing tiny flower buds densely arranged together in a raceme. At this stage, the plant and the inflorescence were given a number respectively. There was no attempt made in this study to determine destructively the timing of floral induction and initiation, because this would lead to a significant loss of replicates in the sample population.

As soon as the inflorescence became exposed, twelve buds were selected from the proximal end, which for the male inflorescence, were at the most advance stage of development. They were tagged with cotton threads of different colours. Parameters such as the length of the peduncle and the length of the inflorescence were recorded weekly. The colour and the size (length and diameter) of each bud/flower/fruit also were recorded weekly. The lengths of the peduncle and axes of male inflorescences were recorded until the last flower withered while for the female inflorescences, they were recorded until fruit maturity. Observations on the colour and size of the male flowers were terminated after the flower withered. The average increases in lengths and diameters of respective parts were determined.

4.2.3 Floral biology

4.2.3.1 Visual observations

All tagged flower buds were observed for anthesis and anther dehiscence/stigma receptivity. The date and time of anthesis, the colour and size changes in floral parts, the date and time of anther dehiscence/putative stigma receptivity and withering were recorded. In the male flower, the parts that were observed for colour and size changes were the anther and the androphore, while in the female flower, it was the stigma. Withering was observed only on the male flower and the colour change of the androphore at the initiation of anther dehiscence and thereafter was used to indicate withering; here, duration of anther dehiscence is the time difference between initiation and withering. The period of 'putative stigma receptivity' however cannot be determined through the changes in colour. This was based on evidence from hand pollination performed throughout this period.

Male flowers were observed at a two-hour interval between 0700 to 1900 hours, daily from the day the first bud opened until the day the last flower withered. Due to the time-consuming nature of these observations, only five inflorescences were used; these inflorescences were not observed on the same plant. The selection of inflorescences was non-randomised. The male inflorescence develops acropetally with the flower buds at the proximal end of the inflorescence opening first and the progressive maturation of flowers on the inflorescence was followed by tagging the flowers that opened during each day. In all observations, each flower was carefully inspected with a hand lens to determine its anther dehiscence time. In *N. macfarlanei*, dehiscence takes place when the anther opens extrorsely with longitudinal splits to release the pollen grains. The flower begins to wither when the androphore changes from light green to light pink.

Female flowers were initially observed at four-hour intervals daily between 0700 and 1900 hours, beginning from the day the first flower opened. Even though the flowers in each inflorescence open simultaneously, only twelve flowers were tagged. The stigma colour at flower opening and thereafter was noted. As more observations took place, it became apparent that the visual colour change in the stigma was extremely slow and so the observations were taken once a week. A total of ten inflorescences were used in this observation throughout the period of study. A few of these inflorescences were borne on a single plant and the rest were from separate individuals. Two or more inflorescences (*i.e.* on two or more plants) were observed simultaneously so as to reduce the time spent on recording these observations, with result that the selection of inflorescences was non-randomised.

4.2.3.2 Observations through the scanning electron microscope (SEM)

Samples of flower at putative receptivity and anther dehiscence were collected in the field, placed in a vial and fixed immediately on return to the laboratory in 4% glutaraldehyde (25% EM Grade, Merck 4239) in 0.1M NaCaCodylate (Natrium Cocadylate, Sigma CO250) buffer. The specimens were shaken gently and left for 4 hours in the fume hood after which the solution was replaced with 0.1M NaCaCodylate. This step was repeated after 15 minutes and the specimens were then stored in 0.1M NaCaCodylate buffer in the refrigerator until required. To prevent the shrinkage of epidermal tissues, specimens were always covered by the solution and were never exposed to air.

The buffer was removed from the vial and replaced with 10ml of 1 NaCaCodylate (0.2M) : 1 OsO_4 (2%) (Osmium tetroxide, Sigma O5500) solution. The specimens were shaken gently, left overnight in the refrigerator and on the following day, they were brought to room temperature. Distilled water was added to dilute the OsO_4 before disposal into a waste bottle. Specimens were rinsed twice for 15 minutes with distilled water.

The specimens were taken through an ethanol dehydration series. The concentrations of ethanol used were 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 100% (x 2), each for 15 mins. The specimens were then placed consecutively into three different combinations of absolute ethanol and acetone, each for 15 mins; the ratios used were 3: 1, 1: 1 and 1: 3, respectively. Subsequently, they were rinsed in three changes of pure acetone (Ajax Chemicals, Clyde Industries, Australia) and brought to the critical point dry using liquid carbon dioxide at 1200 psi. The specimens were sputter-coated with gold particles (JEOL Fine Coat Ion Sputter JFC-1100) and observed under the scanning electron microscope (JEOL JSM-25SII). The floral parts scanned included the surfaces of the

stigma and ovary, the longitudinal sections of the ovary, the surfaces of the dehisced anthers and the pollen grains and the surfaces of the seeds.

4.2.3.3 Floral and fruit production

All plants in the sample plot that produced inflorescences were tagged during the phenological study. In order to determine the ratio of male to female flowers produced during that period and the reproductive efficiency of the female plants, counting was done on all inflorescence produced by the sample population. For the female inflorescence, the numbers of mature and immature flowers were recorded when they reached anthesis while for the male inflorescence, the numbers of mature and immature flowers were recorded after the inflorescence had withered. This was made possible as the flowers were persistent and did not drop from the inflorescence. Similarly, the numbers of mature and undeveloped fruits were noted just before dehiscence. The reproductive efficiency (RE) of the plant was determined using the following formula :

$$\text{Reproductive efficiency} = \frac{\text{Fruit set}}{\text{Floral number}} \times 100\%$$

4.2.4 Embryology

Samples of flower at different stages of maturity were collected and fixed immediately with Craf III fixative (Appendix 14). The specimens were shaken gently and left overnight in a vacuum chamber. The specimens were then removed the following day and stored in a refrigerator in the Craf III fixative. They were then dehydrated using a series of concentrations of tertiary butyl alcohol (Ajax Chemicals, Clyde Industries, Australia) (Appendix 14). At the final tertiary butyl alcohol concentration, the molten wax was added to the vials containing the specimens (Paraplast, Lancer, St. Louis, USA) and placed in a

66°C oven. The molten wax was changed daily for a period of three weeks. The specimens were then embedded in wax blocks. Before sectioning, the wax block was trimmed in order to fit into the holder of the microtome. Sectioning was done at 7 micron and the ribbon was floated on warm water to ease the creases. They were then arranged onto clean glass slides and taken through the staining series using Safranin-O and FCF Fast green before being permanently mounted with Canada Balsam (BDH Laboratory, Poole, England) (Appendix 14) (Johansen, 1940). Male and female buds at different stages of maturity and mature seeds were sectioned.

4.2.5 Flower visitors

4.2.5.1 Activity patterns and frequency of visitors

During the periods of anther dehiscence and 'putative stigma receptivity', the inflorescence was constantly observed for flower visitors. The periodicity, time, duration and frequency of visit and the mode of pollen transfer were noted. The period of observation was from 0900 to 1600 hours and was conducted simultaneously with study 4.2.3.1.

To determine insect visitations at night, observations were carried out for seven nights from 7 p.m. to 7 a.m. the following morning. The flowers were observed at two hour intervals using a penlight. Observations were spread over a period of two months.

4.2.5.2 Foraging behaviour of visitors

The foraging and feeding behaviours of the visitors were observed carefully. Observations at a single period of time were confined to only one inflorescence, as it was not possible to follow the movement pattern of the visitors in the forest. Both male and female inflorescences were observed and as the plant is dioecious, two observers were involved here.

4.2.5.3 Collection of visitors for identification

Samples of flower visitors were caught in a hand net and immobilised with ethyl acetate vapour in vials for subsequent species determination and description. The insects were examined under the microscope for the presence of *N. macfarlanei* pollen grains, after which they were pinned. Any extra samples were placed in 70% alcohol for permanent storage. Methods employed during the collection, preparation and examination of specimens were those of the International Institute of Entomology and the Department of Entomology, Natural History Museum, UK Prior to pinning, photographs were taken either with the aid of a microscope or a zoom lens.

4.2.5.4 Pollen deposition through the scanning electron microscope (SEM)

Pollen deposition on the anatomy of the visitors was also observed using the scanning electron microscope (SEM). Due to the lack of flower visitors, not all species were available for this study. As priority was given to the species determination, only those species with more than two samples were prepared for SEM studies. Such samples were placed in a 40° C oven until required. There were no chemical fixation or dehydration series. Silver conducting paint (Dotite SPI5003-AB) was used to mount the specimens onto 10-mm stubs. The specimens were coated with gold and viewed with the SEM.

4.2.6 Pollination experiments

In order to determine the breeding system of *N. macfarlanei*, pollination studies were conducted. All female inflorescences that were to be manipulated were isolated prior to the opening of the first bud. Three treatments were used, each having five inflorescence replicates:

(i) open pollination. Female inflorescence was tagged for open-pollination.

(ii) control bagging. Female inflorescence was bagged but not manipulated.

(iii) hand pollination. Female inflorescence was bagged and flowers were hand pollinated by applying pollen grains from the male plant.

The criterion for selection of inflorescences in the population was the occurrence of opened flowers during the seventeen months of the reproductive study. Because it was not possible to have 15 inflorescences available at a single period of time for pollination studies, the treatments were staggered throughout the seventeen months. Where possible, five different plants were randomly selected as replicates for each treatment. When this was not possible, one plant was used more than once for the same treatment. In the case of open pollination, the treatment had to be done in two replicates because the first replicate, conducted before September 1994 produced 100 % immature fruits.

4.2.6.1 Isolation

Nylon bags of very fine mesh were used for isolation of the female inflorescences (Plate 5). They were of 0.2 mm mesh size, fine enough to exclude tiny insects and at the same time, sufficiently porous and rigid to allow adequate ventilation. There were no apparent injurious effects on the enclosed inflorescence. Only one inflorescence was enclosed in a bag. Isolation began before the first bud opened and was completed when all the stigmas had turned brown. The mouth of the bag was wrapped securely with a wire and furnished with an inflorescence number. In each of the inflorescences designated for control bagging and open pollination, twelve flowers were selected and tagged prior to isolation. In the inflorescence designated for hand pollination, all flowers were tagged.

4.2.6.2 Application of pollen

Selected flowers in the inflorescence were hand pollinated by brushing pollen from freshly dehisced anthers onto stigmas and then bagged. Hand pollination was done around mid-day

Plate 5 : Isolation of a mature female inflorescence with a nylon mesh bag


5

to accommodate the time spent in travelling from the institute to the site in Genting Highlands. A freshly dehisced flower was removed from a male plant. Application of pollen was done by holding the flower by its pedicel with a pair of forceps and brushing the dehisced anther gently against the stigma. The procedure was repeated with fresh anthers until proper pollination was assured. In order to avoid the uncontrolled entry of pollen during pollen application, the bag was removed from the inflorescence just before application, and during application the inflorescence was carefully watched for any insect visitors.

4.2.6.3 Timing of pollen application

In order to determine the timing of effective pollen application, buds in the female inflorescence were randomly selected and each given a thread tag. Treatments consisted of the different timings of pollen application. Five timings were employed *i.e.* at week two after the first bud opened, at week three, four, five thereafter and control (no application). Due to the lack of inflorescences, only five inflorescences were utilised as replicates in this experiment. The number of buds available for each treatment depended on the total number of buds existing on the inflorescence. The selection of buds for each treatment was done randomly by an individual who had no prior association with *N. macfarlanei.* After each application of pollen, the inflorescence was bagged. Selection of the inflorescence was in a way, randomised because the initiation and development of the female inflorescences in the sample population was not manipulated.

4.2.6.4 Fruit set

Isolation bags were removed after all stigmas had turned brown. Initial fruit set was scored. The fruit size was then progressively scored at weekly intervals until maturity. Thereafter, the number of mature seeds from each fruit was noted. Fruit development was scored for

all flowers used in the hand-pollination treatment and for twelve flowers used in the open and control bagging treatments. The measurements were made by the same individual throughout the period of study. The results were analysed using single factor ANOVA (model II) (Sokal & Rohlf, 1981).

4.2.7 Photographs

Photographs were taken with Nikon 601 and 801 SLR cameras using Kodak 35 mm ASA 100 Colour films. Photomicrographs were taken with an Olympus camera mounted on an Olympus Microscope with Kodak ASA 100 colour film. The films were processed and printed at Kemajuan Laboratories and Kodak Komal Colour Laboratory, Kuala Lumpur, Malaysia. SEM video prints were taken using a video copy processor (Mitsubishi Model P68E) and software developed by JEOL (SemAfore). The thermal paper used for prints was of model K65HM.

4.3 RESULTS

4.3.1 Phenology

The sample population at Mt. Purun consisted of 8.3 % female plants and 5.4 % male plants, the remaining 86.3% being juvenile plants (Table 3). The ratio of female to male plants was 1.5:1. During the phenological period, the ratio of flowering females to flowering males increased to 2.5:1. The significant increase in the ratio indicates that the flowering cues in the population are better synchronised in the females and that the female population is more prolific than the male.

In the Mt. Purun population, males began flowering after they had attained at least two metres in length (HC4) while females began flowering after they had attained at least one metre (HC3) (Table 3). Observations showed that seven female plants in HC3 flowered during the phenological period. No male plants in that category flowered. The categories with the greatest number of flowering female and male plants were 3 and 5 respectively. Although no studies were undertaken to correlate height with age in male and female plants, there is only one apical shoot in the plant and growth occurs along the shoot axis. As such, height of a plant will be correlated to a certain extent with age. The different categories in which males and females flower implied that males begin flowering at a later age than females. Analysis of the results showed that although female plants flowered at an earlier age, the fecundity of these plants, as measured by fruit set, is not significantly different from those that flowered at a later age (Appendix 15). The absence of correlation was derived from a small sample of flowering female plants; it is not known whether a similar relationship would be obtained from a larger sample. The sample population at CH consisted of 0.72% female plants and 0.72% male plants. During the phenological period, 42 % of the female plants and 58% of the male plants were flowering or fruiting. The ratio of flowering females to flowering males was 1:1.4. This ratio differs significantly from that

Table 3 : The number of male and female plants in various height categories in the sample populations at Mt. Purun and CH

Study site		Height Category (HC) *						
		1	2	3	4	5	6	Total
Mt. Purun	No. of plants	101	17	41	24	12	10	205
	No. of males	0	0	0	2	4	5	11
	No. of males flowering during the study period	0	0	0	1	3	2	6
	% flowering in the sample population	0	0	0	0.5	1.5	1.0	3.0
	No. of females	0	0	7	4	4	2	17
	No. of females flowering during the study period	0	0	7	4	2	2	15
	% flowering in the sample population	0	0	3.4	2.0	1.0	1.0	7.8
CH	No. of plants	911	342	203	50	44	117	1667
	No. of males	n.a.	n.a.	n.a.	n.a.	n.a.	12	12
	No. of males flowering during the study period						7	7
	% flowering in the sample population						0.4	0.4
	No. of females	n.a.	n.a.	n.a.	n.a.	n.a.	12	12
	No. of females flowering during the study period						5	5
	% flowering in the sample population						0.3	0.3

* HC 1 = < 0.5 metres high
2 = 0.5 - 0.99 metres high
3 = 1.0 - 1.99 metres high
4 = 2.0 - 2.99 metres high
5 = 3.0 - 3.99 metres high
6 = > 4 metres high

of the Mt. Purun population which was female-biased. This could be due to the selection of plants for phenological observation. The CH plot was primarily an ecological plot and only plants which were greater than 4 metres in length were investigated while at Mt. Purun, because it was a phenological plot, all plants that reached reproductive maturity were investigated. If age is one of the factors influencing the flowering initiation of a plant,

then the sample selection at CH would have missed out many of the flowering females. In addition, the sampling method used at CH was based on a transect while the one used at Mt. Purun was a grid. The inverse ratio at CH is similar with the ratio in HC5 (1:1.5) and HC6 (1:1) at Mt. Purun. This preliminary result is in contrast to the indications that a male-biased sex ratio commonly occurs in dioecious tropical tree populations (Bawa & Opler, 1975).

4.3.1.1 Flowering periodicity

Both sexes of *N. macfarlanei* recorded flowering activity throughout the 100 weeks of phenology (Fig. 13). During this period, fluctuations in flowering were detected in the male and female populations with the female population showing more distinct fluctuation than the male. The flowering observed in the female population suggests some form of periodicity, although the phenological period which spanned almost two years may not be sufficient for this to be confirmed. However, extrapolating before the phenological period, the data obtained in the present fruit production study (Fig. 14) showed that flowering had occurred during 1993 and that a previous peak had occurred early in that year. Therefore, it does seem likely that some form of periodicity exist in the female population although it is difficult to determine at this present time what the causes and significance of such fluctuations might be. Studies on the pattern of flowering in tropical species have shown that changes in the environment, the availability of pollinators and the interspecific competition for pollinators are critical factors determining the timing and periodicity of flowering (Bawa, 1983). It is quite possible that these factors also apply to the flowering of females in *N. macfarlanei*. In contrast, the male population did not show as distinct the fluctuations in flowering although the intensity was higher in some months compared to other months (Fig. 13).

Fig. 13 : The number of female and male inflorescences at anthesis in the sample population at Mt. Purun during August 1993-July 1995

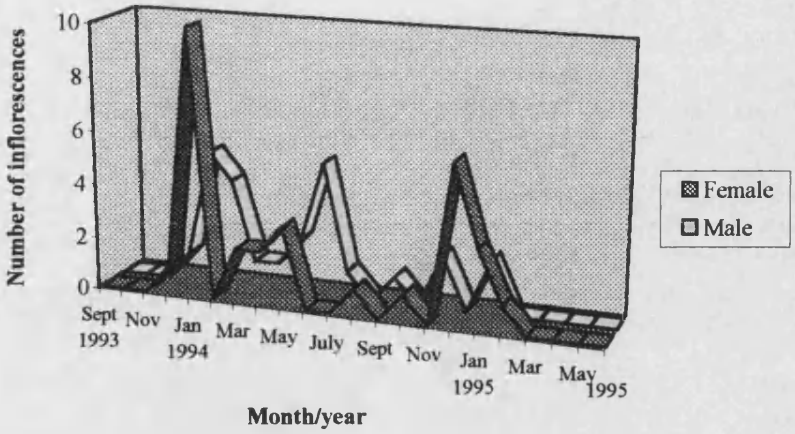
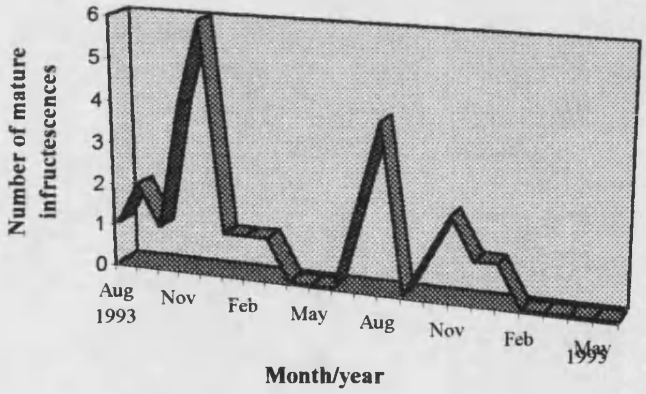


Fig. 14 : The number of mature infructescences in the sample population at Mt. Purun during August 1993-July 1995



In the female population, two peak flowering periods were observed (Fig. 13). The first peak was from early November 1993 to May 1994 and this was followed by receptivity from early January to early July 1994. The second peak occurred from early November 1994 to March 1995, followed by receptivity from mid January to late May 1995. Although no receptivity was observed in between those months, young buds and fruits were developing in the sample population.

In the male population, flowering was more dispersed throughout the period compared to the females and peak periods were less distinct (Fig. 13). Flowering was recorded in December 1993 and there was activity throughout the phenology period. This activity intensified from early January to late August 1994. Anthesis took place every month in1994 but it reduced after the month of August. No anthesis occurred after March 1995. Fig. 13 clearly shows that the anthesis period in the male population coincided with that of the female population.

Similar to flowering, fruiting occurred throughout the phenological period (Fig. 14). Mature fruits were available in the population as early as August 1993 and the fruit maturity attained a peak in the early period of phenology where at least 509 mature fruits were collected from the population. This number was by no means small, indicating that flowering had occurred several months earlier for the female population and that they may have undergone a peak period in receptivity. The periodicity in fruiting during the phenological period was not fully representative of the sample population based on obvious reason. As many of the mature inflorescences produced during the study period were experimentally manipulated, the subsequent infructescences were not included in the graph.

In CH, the flowering of *N. macfarlanei* was sporadic, in contrast to the flowering pattern at Mt. Purun. This could largely be due, however, to the sampling method and the distance of the study site, which did not enable the author to conduct more regular monitoring. Apart from January-April 1994, flowering and fruiting occurred in all the remaining months (Appendix 16).

4.3.1.2 Flowering intensity

During the phenological period, 100% of the female flowers in the population reached anthesis. 55.1 % of the female flowers in the population were receptive between early January and early July 1994 (the first peak period) (Appendix 21b), and 35.5% were receptive in January and February. 21.9% of the female flowers produced receptive stigmata between early January and late May 1995 (second peak period), and 12.9% were receptive in January and February. The total percentage of flowers maturing in both peak periods was 77%; the remaining flowered in between peak periods. The flowering intensity, therefore, reached its peak only once in a calendar year, between January and February.

During the phenological period, 93% of the male flowers in the population reached anthesis (Appendix 21a). 82.1% were receptive between early January and late September 1994, and in this period, 28.7% were at anthesis in January and February (see also 4.3.3). Even though flowering took place in 1994 and 1995, there was not enough evidence to show that certain years would be more intensive that others.

During the phenological period, only 59.4% of the total female flowers in the population reached fruit maturity (Appendix 21c). This did not include the fruits developed from pollination treatments.

The flowering intensity at CH was difficult to determine for the reasons given above. However, it is clear that the peak period of flowering did not fall in the months of January and February, at least for 1994 (Appendix 16).

4.3.2 Flower and fruit development

The mean number of buds in the male inflorescence was 122.4 ± 32.97. Anthesis began before the axes had attained its maximum length (mean length 29.0 ± 5.8 cm) and by the time the last flower had withered, the inflorescence had acquired a mean length of 33.27 ± 8.2 cm. In contrast to the male inflorescence, the female axes was shorter at maximum length (22.0 ± 4.5 cm) and the mean number of buds was 46.7 ± 15.19; anthesis began only after the axes had attained maximum length and this length was maintained until fruit maturity. This is consistent with observations made by Danser (1928). Although no determinations could be made on the inflorescences in the sheaths without the loss of the inflorescences as subsequent experimental units, observations have shown that because of the large difference in the number of buds, male inflorescences are larger than the female inflorescences from an early stage (Plates 3a & 3b). The size difference can, therefore, be used to predict accurately the sex of a plant even before its inflorescences emerge from the sheaths.

4.3.2.1 Flower development

In the male inflorescence, the first bud required a mean of 5.4 ± 1.14 weeks before it reached anthesis (Appendix 17). Thereafter, mature flowers were produced in small numbers continuously up to a mean of 5.4 ± 1.34 weeks with the range of between 4-7 weeks. Anthesis occurred at any period of the day and it generally required another 24 hours to reach anther dehiscence; some flowers were observed to require 36 hours. Data collected from 281 flowers showed that anther dehiscence took place between 0630-1700 hours and there was no dehiscence at night (Appendix 18). Dehiscence time apparently varies between the highland and the lowland species. The anther dehisced longitudinally to expose the pollen grains. The grains are glossy yellow in colour and the exine markedly

corrugated with ovate-triangular spines (Plate 6). Morphologically, the pollen of *N. macfarlanei* is similar to *N. alata*, *N. pyllamphora*, *N. vieillardii*, *N. lowii* and *N. villosa* (Erdtman, 1972). Observations on the pollen deposition on the body of captured visitors showed most of the grains were present in clumps. Several screenings of the receptive stigma surface with the SEM showed that each grain in the tetrad is probably able to germinate and produce a pollen tube (Plates 7a & 7b), since the presence of other grains within the tetrad is apparently not an inhibiting factor to pollen tube germination. This ability can probably be considered to provide a competitive advantage for a species such as *N. macfarlanei* where intraspecific and interspecific gene flow and the acquisition of illegitimate pollen grains are likely to be high, as evidenced by the presence of other *Nepenthes* sp. at the same site and the production of interspecific hybrids.

Anatomical sections of young buds showed that the anther sac is already densely filled with pollen mother cells (PMCs) (Plate 8). The tapetal cells are no longer in distinct layers but have begun to disintegrate. The connective tissue is made up of parenchyma cells with densely staining bodies. The PMCs may be present either as a single cell or as many cells adhering together. They have dense cytoplasm and a prominent nucleus. By the time the bud reaches 0.4x0.5 cm, the tapetal layer has completely disintegrated. Because the PMCs exist in clumps often resembling tetrads, it was difficult to determine the stage where the division into haploid cells began.

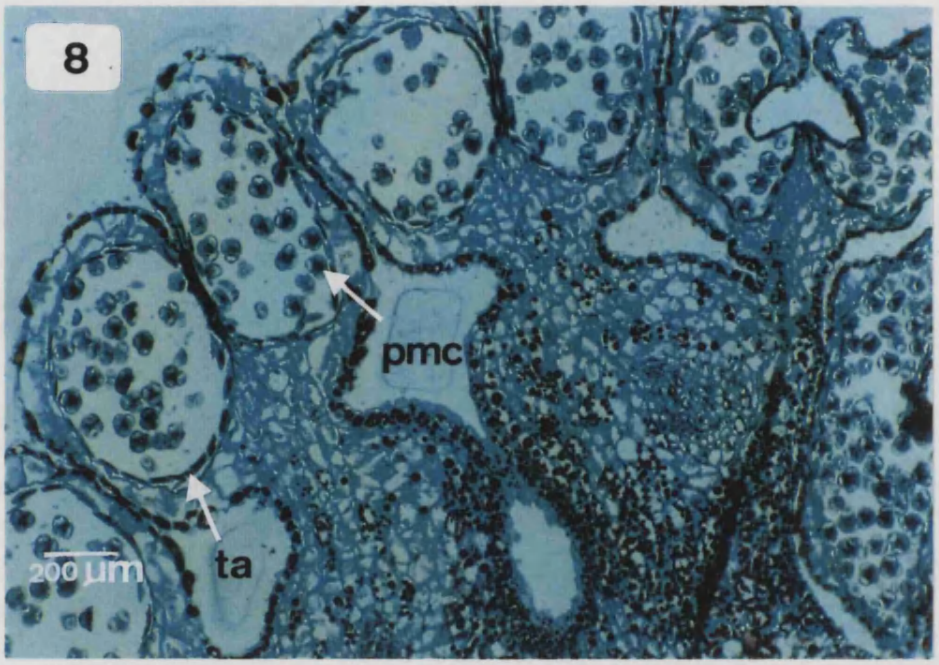
As for the female inflorescence, it required a mean of 7.2 weeks after exposure to reach anthesis which may occur at any period of the day (Appendix 17). At anthesis, the stigma is green, glistening and presumably receptive while the ovary is brown and densely covered with long adpressed hairs (Plate 9). The advantage arising from the ability of each pollen

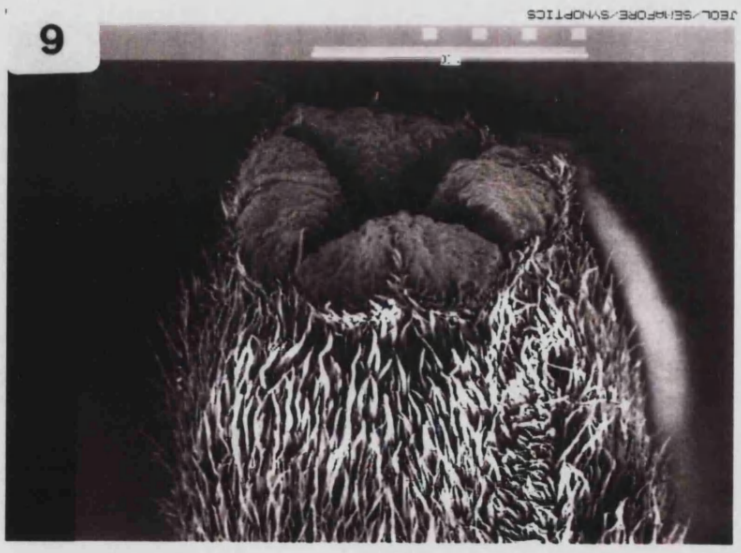
Plate 6	:	The exine of the pollen grain of *N. macfarlanei*. Magnification x1500; scale bar in micron.
Plates 7a - b	:	The germination of pollen tube (pt) in *N. macfarlanei*. Scale bar in micron.
Plate 7a	:	The pollen tube development. Magnification x1500.
Plate 7b	:	The development of more than one pollen tube within a pollen tetrad. Magnification x2000.


6
JEOL/SEM4FORE/SYNOPTICS
FRIM


7a
pt
JEOL/SEM4FORE/SYNOPTICS
FRIM


7b
pt
JEOL/SEM4FORE/SYNOPTICS
FRIM


8
pmc
ta
200 µm


9

grain to germinate within a tetrad is probably counterbalanced by the loss of legitimate pollen grains through its deposition on the hairs of the ovary as the pollinators forage among the flowers.

Anatomical studies on young female buds showed that at anthesis, the micropyle region was distinct and there were mitoses taking place in some of the archesporial cells although no functional megaspore mother cells (MMCs) were observed (Plate 10). Sections taken from a more advanced anthesis stage (the fully reflexed tepal stage) showed the presence of functional MMCs with clearly-defined inner and outer integument layers and nucellus tissue (Plate 11). Nuclear activity, presumably related to meiosis, could be detected in some of the MMCs (Plate 12). It was observed that the timing of development of the megaspore differed between ovules in a single ovary; therefore, it is also likely that there is variation in the timing of development of megaspores between flowers in a single inflorescence. The asynchronous development in the ovules in a single ovary has also been reported for *N. gracilis* (Lim & Prakash, 1973).

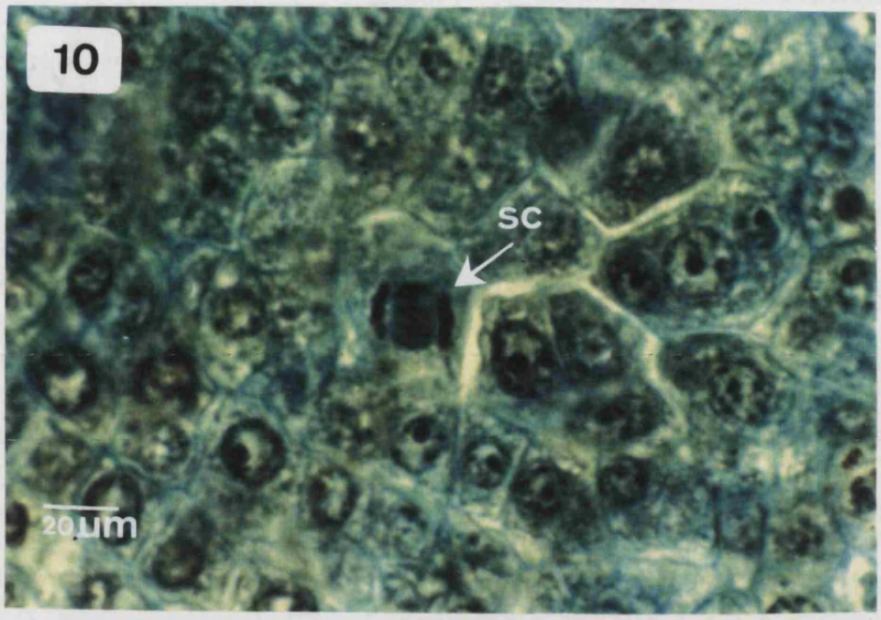
4.3.2.2 Fruit development

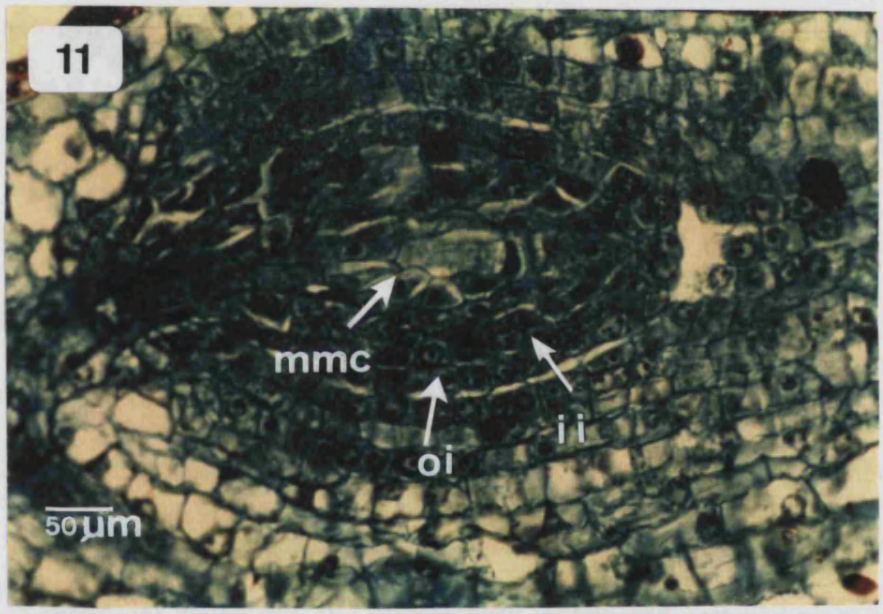
The developing capsule is greenish brown in colour, later maturing brown. The fruit required a mean of 23.2 ± 3.24 weeks to reach maturity. The increment in fruit length was stepped and the mean size at dehiscence was 2.2 x 0.5 cm (Appendix 19). Fruits in an infructescence reached the dehiscence stage simultaneously. This implied that although the timing of the development and fertilisation vary between ovules, the development of embryos is fairly well synchronised between ovaries.

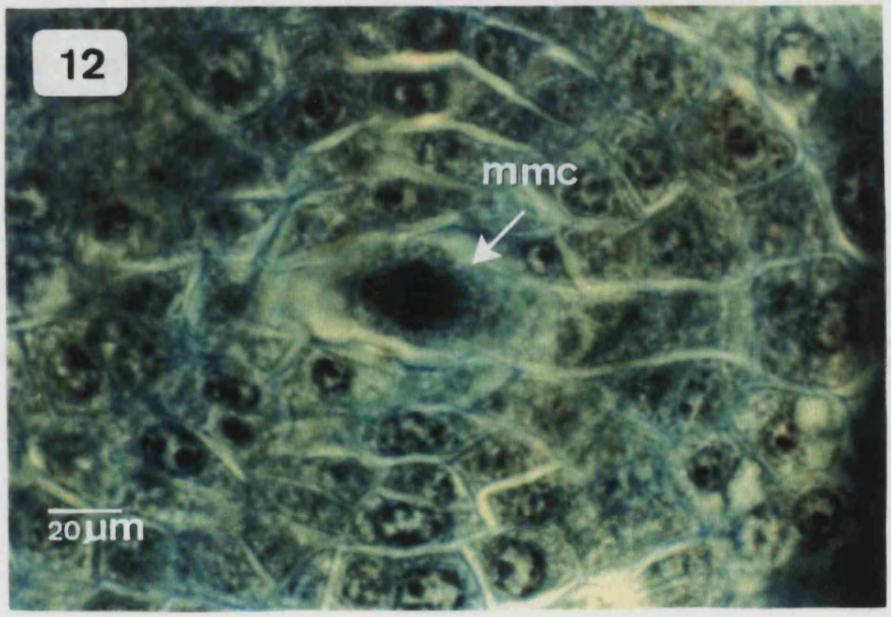
Plate 10 : Longitudinal section of an ovule showing the mitotic activity in the sporogenous cells (sc). Magnification x100.

Plate 11 : Longitudinal section of an ovule showing a functional megaspore mother cell (mmc) bearing nucellus tissue and inner (ii) and outer integument (oi) layers. Magnification x40.

Plate 12 : Longitudinal section of an ovule showing nuclear activity, presumably related to meiosis, in a megaspore mother cell (mmc). Magnification x100.


10
sc
20µm


11
mmc
oi
ii
50µm


12
mmc
20µm

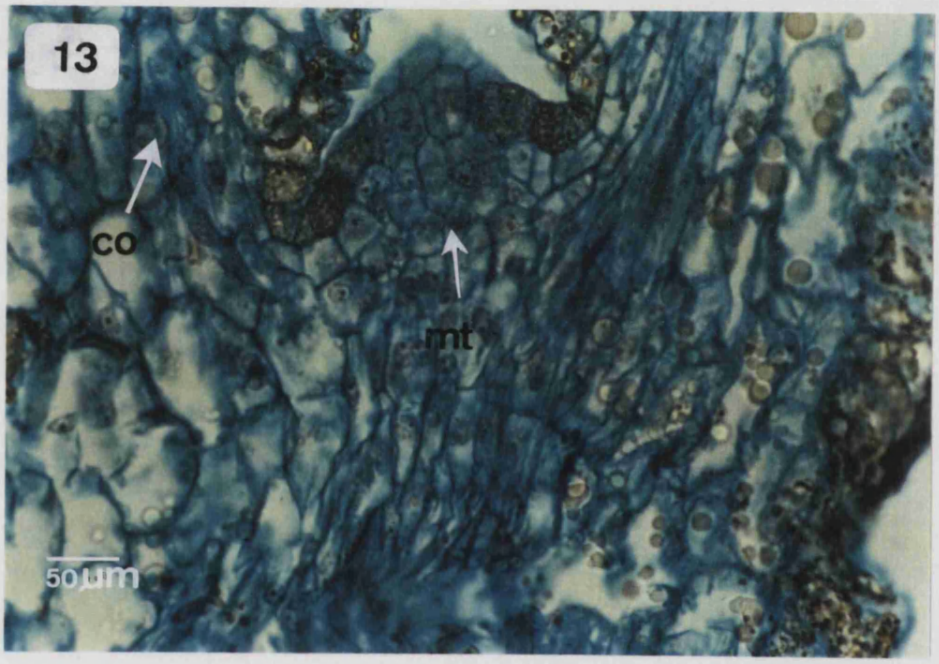
Danser (1928) and subsequent workers have suggested that the seeds are wind-dispersed. Although no direct study was undertaken to determine the mode of dispersal, field and anatomical observations showed that the seed is characteristic of a wind-dispersed species. It is small, filiform and very light. The anatomical sections of the mature seed showed that much of the tissue in the embryo consisted of large, vacoulated parenchyma cells (Plate 13) and the entire embryo occupied only a small central portion of the seed; the rest being seed coat. The size of the embryo is a mere 2.0 x 1.0mm and it is doubtful that an embryo of that size would be particularly attractive to animals. The anatomy and morphology of the embryos is similar to those reported by Lim & Prakash (1973) and Kaul (1982). They have two cotyledons and a hypocotyl and the cotyledons have large parenchyma cells with numerous vacuoles. The hypocotyl region consists of smaller cells with visible cytoplasm and nucleus.

4.3.3 Anther dehiscence and 'putative stigma receptivity'

During selected dates in the period 30 December 1993 to 9 Mac 1994, there was at least one male flower at the dehiscence stage in an inflorescence (Appendix 20). The number ranged from 1-8. The number of dehisced anthers varied between dates and between plants. Even though there was no record of a flower with undehisced anther during the selected dates, the possibility that it occurred during other days cannot be ruled out.

At the population level, there was at least one flower recorded at the dehiscence stage on each date (Appendix 20) and this number was recorded in 18.6% of the total number of days. In contrast, the highest record of dehisced anthers per day was 29 and this happened on the 2 March 1994. The mean number of anthers dehiscing in a day in the sample population was 5.7, the actual number ranged from 1-29. Again, the possibility that none

Plate 13 : Longitudinal section of a mature embryo showing the meristematic region (mt) and cotyledons (co). Magnification x40.

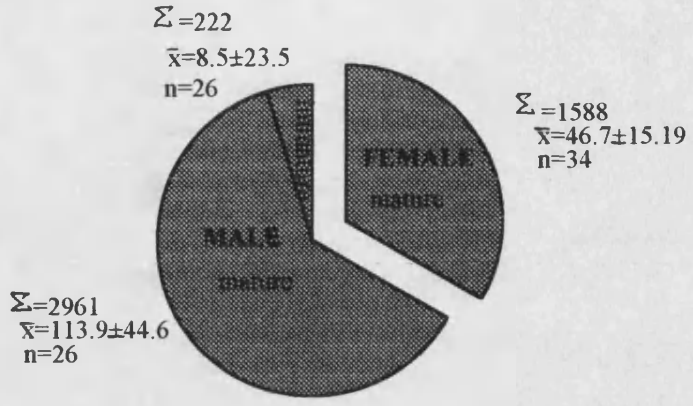

13
co
mt
50um

dehisce during other days in the flowering season cannot be ruled out and, similarly, the population may produce more than 29 dehisced anthers in a single day.The total number of dehisced anthers recorded in the sample population during the 41-day observation period was 233 (Appendix 20). 48.5% of this number dehisced between 11 January-8 February 1994. Between the same period, three female inflorescences produced a total of 89 receptive flowers (Appendix 20). In absolute numbers, there were 89 receptive flowers to at least 113 dehisced ones in the sample population during 11 January-9 February. This gave a ratio of 1.3 male flower to each female flower. This ratio is likely to fluctuate during the flowering season.

4.3.4 Floral and fruit production

The total number of male flowers produced in the sample population from December 1993 to March 1995 was 3183 (Fig. 15). The mean number of inflorescence produced by a plant during this period was 2.5 with the actual number ranging from 1-5 (Appendix 21a). In all the males sampled, only one (*i.e.* 05) produced five inflorescences. It was more common to find plants producing a maximum of three inflorescences. The mean number of flowers per inflorescence was 122.4 ± 32.97 with a range between 66-198. Only one male in the sample population (*i.e.* 09) consistently produced flowers below the lower limit. In comparison, males 04, 05 and 07 were more vigorous and fertile than male 09. However, in the case of male 05, almost 75% of the flowers in inflorescence 05 did not develop to maturity and in the case of male 07, 100% died in inflorescence 04. This is hardly surprising as higher production will affect the floral resource allocation in the plant with the result that in certain plants the last inflorescence produced will be less vigorous. There was great deal of variation between plants in terms of their abilities to produce inflorescences in this phenological period and this was possibly due to the genetic heterogeneity that is derived from outcrossing.

Fig. 15 : Floral production in the sample population at Mt. Purun during August 1993-July 1995



n= number of inflorescences

91.1% of the male flowers produced in the population reached the stage of anther dehiscence (Appendix 21a). The remaining 8.9% were distributed between 41% of the sampled plants, each of which only had a small percentage of undeveloped buds. Most of these buds were positioned at the far distal end of the inflorescences and the long maturation period experienced by the inflorescence would probably have exhausted its floral resource.

The female population produced significantly less flowers compared to the male population (Fig. 15). During August 1993-July 1995, a total of 1588 flowers were produced in the sample population. The mean number of flowers per inflorescence was 46.7±15.19 with a range between 17-82. The mean number of inflorescence per plant was 1.5 with a range between 1-4 (Appendix 21b). Only two females produced up to three inflorescences. At least four inflorescence died in sheath compared to none in male plants. All these were the last in the series produced by the plant during the period of phenological study. Excluding the four inflorescences that died in sheath, 100% of the flowers produced by the female population reached anthesis stage.

Although 100% of the female flowers in the sample population attained reproductive maturity, only 59.4% developed into mature fruits (Appendix 21c). The reproductive efficiency (RE) of the sample population was 59.2 ± 39.99%. The RE for an inflorescence vary considerably between 0 and 100%; in almost all cases, it declined from 100% to 0% as more inflorescences were produced by the plant. The mean RE for an individual female plant ranged from 27.4 to 95.1%. Only 58% of the sample individuals had RE greater than the RE of the population. Regression analysis showed that there is no relationship between the nearest male distance with the RE of female plants in this population, indicating that a female plant is not dependent on the nearest male for high fruit set (Appendix 22). This has implications to the intraspecific and interspecific gene flow in *N. macfarlanei*. As will be shown presently, this species is entomophilous and its pollen flow is mediated by a diverse group of small insects. The lack of relationship between the nearest male neighbour and RE of the female population suggest that its gene flow through pollen is constrained only by the size of the patch within which the potential pollinators forage.

A mature fruit produced a large number of seeds. In open pollinated flowers, the mean number of mature seeds was 92.5 ± 14.54 while the mean number of immature seeds was 44.2 ± 15.17 (Appendix 21d). A random sample of 20 fruits gave a total number of 1850 mature seeds, this being 67.7% of the total number of seeds. Although the combination of fruit set (RE=59.2%) and seed set (67.7%) during the period of observation was not particularly high, the cumulative number of seeds produced by each plant during its lifespan is likely to be high. For example, even during the phenological period, assuming the RE per plant was the same as the RE of the sample population and that a plant produces 1.5 inflorescences during one flowering period, the total number of mature seeds provided by the plant would be 5458 and the total number of mature seeds available in the sample population during the same period would be 321,992. If similar amounts are also produced

by other populations, this is likely to enhance the long-term regeneration, the breeding capacity and ultimately the survival of the species.

4.3.5 Flower visitors

In general, there was poor insect visitation on the mature flowers of *N. macfarlanei* during the 15 days of observation. Only 9 days had some visitation, the remaining days had none (Appendix 23). This observation is consistent with those made by Kato (1993) on *N. gracilis*. Although no climatic data were collected throughout the period of observation and therefore no direct correlation can be made with pollinator activity during those days, most of the days were foggy, wet and windy with sunlight available only for a few hours during the later part of the morning and early afternoon (personal observations). The generally cloudy weather could perhaps have influenced the frequency of visitations. If such low frequency occurs throughout its flowering period, severe competition for legitimate pollinators between the flowering individuals will exist, resulting either in the abortion of unfertilised ovules or poor seed set or both.

During the period of observation, male and female inflorescences were visited by wasps, flies, bees, plant hoppers, ants, cockroaches, spiders, mosquitoes and crabs (Appendix 23). Among the potential pollinators, Diptera (flies) were the most commonly encountered visitors; these included Syrphidae (flower flies), Muscidae (houseflies) and carrion flies (family not determined) (Plate 14a-c). Other potential pollinators included Tiiphidae (tiiphid wasps), Halictidae (halictid bees) and Dictyopharidae (plant hoppers) (Plate 15a-d). These insects can be considered to belong to the group of generalists because apart from *N. macfarlanei*, they have been documented on flowers of unrelated plant species (Proctor & Yeo, 1973; Faegri & van der Pijl, 1979). Formicidae (ants) were frequently observed on the male and female flowers of *N. macfarlanei* but because they are more often considered

Plates 14a - c : The Diptera group of flower visitors observed foraging on the male and female flowers of *N. macfarlanei*

Plate 14a : Muscidae (housefly)

Plate 14b : Syrphidae (flower fly)

Plate 14c : Carrion fly (arrow; family indet.). Note the presence of pollen grains of *N. macfarlanei* on its anatomy

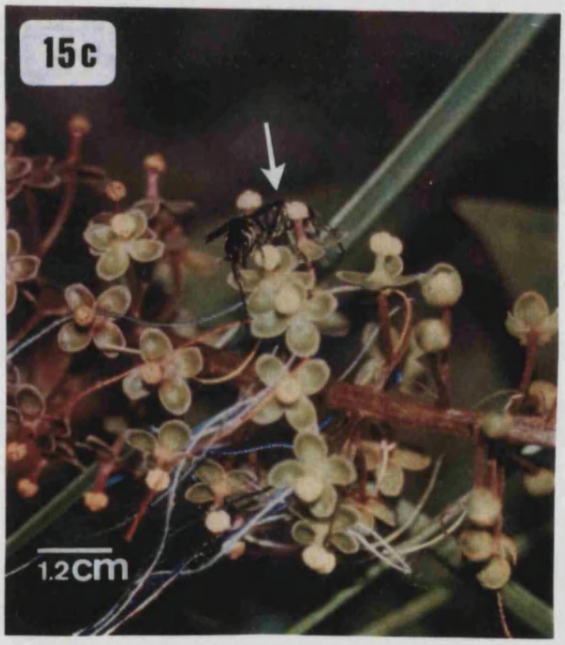

14a
0.5CM


14b
0.5CM


14c
1.0 cm


15a
0.5CM


15b
0.3CM


15c
1.2cm


15d
0.025CM

as nectar thieves than potential pollinators (Faegri & van der Pijl, 1979), their role here was not investigated. Similarly, cockroaches, spiders and mosquitoes are not considered to be potential pollinators because their foraging nature do not seem to be an effective mode for pollen transfer.

Although the male flowers were visited by all the above potential pollinators, syrphid flies and halictid bees made more visits compared to tiiphid wasps, muscid and carrion flies and plant hoppers (Appendix 23). Out of the 11 observed visits on the male flowers, four and three were those by syrphid flies and halictid bees respectively, the remaining four were distributed between carrion fly, muscid fly, tiiphid wasp and plant hopper. While a syrphid fly visited the same inflorescence three times within a span of 30 minutes, the halictid bees visited different plants on separate days. Apart from the syrphid fly and halictid bees, other insects did not visit the male flowers frequently, at least during the period of observation.

Insects that visit both the receptive male and female flowers are more likely to be pollinators than those that visit flowers of one sex only. In the case of *N. macfarlanei* at the sample plot, only muscid flies were recorded on both the male and female flowers (Appendix 23). Although only one visit was recorded on the male flowers during the observation period, five visits were made during the same period on the receptive female flowers; many of these visits were on the same inflorescence and three occurred within the span of 40 minutes. This species seemed to have an affinity for female plants for it was also observed visiting the nylon bag of one receptive inflorescence and the fruits of another female plant. These observations suggest that muscid flies are pollinators for *N. macfarlanei*, although at this stage, as explained above, it is premature not also to consider syrphid flies and halictid bees as potential pollinators for *N. macfarlanei.*

Detailed observations of the foraging pattern of a muscid fly on receptive flowers showed that pollen deposition, either from the same species or from other species, can easily occur. The fly clambered from flower to flower in circular motions, foraging on the inner tepals and the ovary. This type of movement enhances the amount of pollen deposited on the first few stigmata visited, this becoming gradually less as more flowers were visited. This could perhaps explain why some of the open-pollinated flowers did not develop into mature fruits, as detected from the pollination experiments conducted by the author.

Lim & Prakash (1973) mentioned that in *N. gracilis*, the anthesis time is accompanied by a heavy foetid odour; similar observations were made by the author with *N. macfarlanei*. It is interesting to note that although butterflies and moths (Lepidoptera) were observed in the area on certain days and although they were less than 2 metres away from mature flowers, they never visited the flowers. Perhaps the flowers did emit a type of smell attractive to insects from the Diptera group only. This observation is in contrast to that of Kato (1993), who reported that moths were the most common visitors in *N. gracilis*.

4.3.6 Pollination

4.3.6.1 Fruit set

Flowers in the control bagging treatment did not develop into fruits (Table 4). In open pollination, slightly more than half of the flowers had successful fruit set while in hand pollination, 50% of the treated flowers managed to reach fruit maturity. In the open pollination treatment, five developing infructescences died without reaching maturity; all of them were from different plants. While the death in control bagging was associated with the death of the infructescence, none of the infructescences in the hand pollination treatments died. Even though there was post floral development in all treated flowers (see below), there was no indication of apomixis as all the flowers in control bagging and the flowers

not applied with pollen grains in the hand-pollination treatments (controls) did not develop to mature fruits.

The mean number of mature seeds per fruit developed from hand-pollination was lower compared with that resulting from open pollination, indicating that insufficient pollen grains had been applied. The percentage of mature fruits and the mean number of weeks taken to reach maturity were, however, comparable for both treatments (Table 4).

Table 4 : Fruit-set in inflorescences selected for pollination treatments

	Open pollination	Control bagging	Hand-pollination				
			week 2	week 3	week 4	week 5	no application
No. of flower replicates	60	60	20	20	20	20	19
No. of plants	5	5	4	4	4	4	4
% of mature fruits	57.3	0	35	10	15	0	0
% of immature fruits	42.7	100	65	90	85	100	100
Mean no. of weeks to reach fruit maturity	23.2	-	19	18.5	18.3	-	-
Range of weeks to reach fruit maturity	17 to 30	-	18-20	18-19	18-19	-	-
Mean no. of mature seeds	92.5 ± 14.54	0	78.1 ± 59.26	75.3 ± 45.6	71.7 ± 50.2	0	0

NB : - = not applicable

The post-floral development was recorded for all treated flowers. The post-floral growth began with the increase in the size of the ovary after the stigma had turned brown. The increment was stepped and it increased beyond 1.0 cm. However, fruits that were not successfully fertilised never developed to maturity and, even though the unfertilised seed possessed a seed coat, these fruits never contained well-developed seeds.

4.3.6.2 Timing of pollen application

ANOVA results showed that the timing of pollen application is highly significant ($P<0.001$) (Appendix 24) with the mean fruit length following pollination at week 2 differing significantly from the mean fruit length following pollination at other times. The highest receptivity of the stigmata during the experimental period was at week 2 after anthesis; some of the stigmata were still receptive after two weeks as evidenced by the higher variation in fruit length produced by flowers pollinated at weeks 3 & 4. This variation could arise from four factors : first, some of the stigmata could have remained receptive up to a maximum of 4 weeks; second, it could have been due to the lack of pollen applied to the stigma; third, it could have been due, to a smaller extent, to intrinsic variations in the plant possibly reflected in the different development stages of the ovules at the time of application, as seen from the embryological observations (see 4.3.2.1); and fourth, selections of inflorescences for treatments did not omit those produced during the later part of the phenological study. No doubt these factors interacted to determine the fruit set of the plant. Colour changes in the stigma has often been used as indicator of receptivity (Lim & Prakash, 1973; Chan, 1977; Appanah, 1979; Kaul, 1982); in the case of *N. macfarlanei*, this did not accurately reflect the duration of receptivity, as evidenced by the reduced fruit set produced by flowers that were pollinated after two weeks of anthesis. Although no pollination was attempted at the beginning of anthesis, it is not presumptuous to suggest that, in *N. macfarlanei*, highest pollination success can be expected within the first two weeks of anthesis.

4.4 DISCUSSION

4.4.1 Flowering periodicity

Flowering of *N. macfarlanei* in the sample population was observed to occur throughout the year. Although not all mature plants were recorded flowering during the study period, flowering is fairly synchronous, particularly among the female individuals in the population. There appear to be some periodicity in flowering so that the most intensive months are likely to be January and February (Fig. 13). Both male and female flowers reached anthesis stage simultaneously; this is clearly an advantage for *N. macfarlanei*, in which the breeding system is allogamous, since there are always likely to be some male and female flowers within the population reaching the anthesis stage concurrently.

Phenological changes in a plant represent adaptations to abiotic and biotic factors (Medway, 1972; van Schaik *et al.*, 1993). In the case of *N. macfarlanei*, abiotic factors may determine the flowering periodicity whereas biotic factors may determine the duration and intensity of flowering as suggested by a fairly general rule for tropical flowering plants (van Schaik *et al.*, 1993). Abiotic factors such as the changes in microclimate (Holttum, 1940b; Schwabe, 1971; Opler *et al.*, 1976; Ng, 1984; van Schaik *et al.*, 1993; Young & Mitchell, 1994; Aizen & Feinsinger, 1994a) and global climate (Ashton *et al.*, 1988; Glynn, 1988) and biotic factors such as the seasonality in the availability of pollinating agents and the resulting competition for pollinators (Rathcke & Lacey, 1985; Wheelwright, 1985; Bawa, 1990) are likely to influence its pattern of temporal clumping phenology. From the compilation of 53 phenological studies on various species in tropical climates, van Schaik *et al.* (1993) showed that community peaks in flowering were closely related to the peaks in irradiance. At the phytogeographical scale, Ashton *et al.* (1988) have found, for the Dipterocarpaceae, a broad correlation between mass flowering seasons at different localities with the El Nino climatic events in the Pacific. The phenological dependence of

N. macfarlanei on climatic factors is not known but it is clear that the flowering patterns in the female and male populations vary. van Schaik *et al.* (1993) and Aizen & Feinsinger (1994b) attempted to explain the interrelationship between phenology and climate by describing a number of hypotheses, from which the one most likely to be relevant to this species is the one based on the effects of climate on the agents of pollination (see 4.4.3).

While the sample population at Mt. Purun had a distinct flowering periodicity, the sample population at CH did not. There could be several reasons for this. Firstly, it could be due to the sampling approach used in CH which was unable to provide sufficient information. Secondly, it could be due to the different microclimates experienced by the two populations; for example, the vegetation at Mt. Purun received higher amounts of rain but it experienced lower relative humidity than the vegetation in CH (Figs. 5a & 5b). Although data on the irradiance levels at Mt. Purun were not available and therefore strict comparisons cannot be made, it can be assumed that the irradiance levels vary between the two sites. The weather pattern is largely intrinsic but the land use patterns in the study areas are likely to influence the microclimate by the creation of edge effects (Wales, 1967; Dierschke, 1974; Williams-Linera, 1990). Although much of the land in CH is under agriculture, it is at least under cover while in GH, it is under resort development and totally disturbed. The degree and extent of edge effects on the vegetation in the forest fragments are likely to vary with the size (Young & Mitchell, 1994) and shape of the fragments (Laurence & Yensen, 1991) and with the vegetation and its age (Gysel, 1951; Bruner, 1977; Ranney *et al.*, 1981). The size and shape of the forest fragments and age of the vegetation at both study sites are not known but it is obvious that the population in Mt. Purun is hardly 1 km away from development activities and therefore it would be under a significant influence of edge effects as described for fragmented forests (Wales, 1967; Kapos, 1989; Matlack, 1993; Young & Mitchell, 1994). The population in CH is much

further away and thus would be under less influence. In addition to the influence on flowering pattern, Aizen and Feinsinger (1994a) have demonstrated that habitat fragmentation does affect plant reproduction and that plants with different pollination requirements might differ in their sensitivity to fragmentation. They, and several other workers, noted that the frequencies of native insects such as bees, wasps and flies decrease from continuos forest to small fragments (Lovejoy *et al.*, 1984; Klein, 1989; Aizen & Feinsinger, 1994b). This could apply to the insect population at Mt. Purun and perhaps partially explained the scarcity of insects during observation days.

4.4.2 Breeding system

The allogamous breeding system in *N. macfarlanei* results in outcrossing and increases the opportunities for intraspecific gene flow between conspecific populations. The resulting high levels of heterozygosity will buffer the population from the effects of genetic drift (Soule, 1980; Simberloff, 1988). Although gene flow is often considered beneficial because it is able to reduce inbreeding depression and depletion of genetic variation (Wright, 1931; Allendorf, 1983; Huenneke, 1991; Lande, 1992), Ellstrand & Elam (1993) have argued the possibility of deleterious effects arising from it particularly when population sizes are small. In the case of the sample population at Mt. Purun, it is not known whether a population of approximately two hundred individuals is genetically small because measures of fitness and levels of genetic diversity were not carried out. Levels of heterozygosity and genetic variability would be expected to vary between populations due to selection pressures in the form of genetic, biological and physical factors. As for interspecific gene flow, the presence of natural hybrids is the clearest indication that it does occur in *N. macfarlanei* and in other *Nepenthes* sp. Such introgression may bring benefits but it may also place the species at risk from genetic assimilation (Ratcliffe, 1973) and outbreeding depression (Ellstrand & Elam, 1993).

The long receptive period in the female flower of *N. macfarlanei* is rather unusual when compared to that in other species (see 4.3.6.2) and it is most likely to be related to the frequency of visits by pollinators. If the low frequency observed during the period of pollinator study is a common occurrence throughout the duration of flowering in the sample population (see 4.3.5), it can only be expected that the female flowers remain receptive for a longer period so as to increases the competitiveness for pollinators and opportunities for mating. Interestingly, Primack (1985) and Stratton (1989) have found that in general, flowers in the cloud forests have a longer life span than those in the lowland rain forests and Primack *loc. cit.* and Bawa (1990) found this to be consistent with the notion of increased competitiveness in face of the unpredictability in relation to pollination.

The ratio of male flowers to female flowers produced by the sample population during the phenological period was 2.6:1 (Fig. 15). This is perhaps not unexpected for male plants in dioecious species are known to bear more flowers than female plants (Opler & Bawa, 1978) and that males tend to optimise the quantity of matings while the females tend to optimise the quality (Ghiselin, 1974; Williams, 1975; Janzen, 1977). Perhaps by producing flowers steadily but eventually summing up to a large number at the end of anthesis, the female flower may receive sufficient pollen grains for fertilisation. The acropetal development in the male inflorescences together with the synchronous development in the female inflorescence is likely to be an adaptive feature in the sexual selection of the species.

4.4.3 Pollination syndrome

There have been many deliberations on the pollination mechanism of *Nepenthes* sp. Past research has strongly suggested insects as the pollinating agent (Lim & Prakash, 1973; Kaul, 1982; Adam *et al.*, 1989). The present results from pollination studies and field observations provide ample evidence to support this hypothesis, at least for *N.*

macfarlanei. Firstly, several diverse groups of small insects visited the mature male and female flowers. Although no attempt was made to determine the nectar production during anthesis, regular observations using a hand lens clearly showed the presence of nectar on the tepals of flowers and the stigma throughout this period. The nectar and pollen grains are food sources, making the flowers attractive to insects (Sprengel, 1793). The long period of anther dehiscence/stigma receptivity also implies that the flower remains attractive for the same period of time. Secondly, microscopic observations on the body of captured visitors clearly showed that they carried pollen grains. These grains are often in large clumps. Thirdly, the anther dehiscence is diurnal and coincided with the higher insect activity observed during the day. Fourthly, the inflorescence is rigid, held upright and in climbing individuals, it often protrude out above the foliage (Danser, 1928; Kurata, 1973; personal observations). The flower is erect with a convenient landing place for flying insects and it exposes its floral rewards for a long period. These floral and pollen morphologies are typical of an insect-pollinated flower (Proctor & Yeo, 1973; Faegri & van der Pijl, 1979). Finally, the female flowers in the control bagging treatment died after anthesis. The use of isolation bags has not been reported to prevent the transfer of pollen through wind (Bawa & Crisp, 1980) but in the case of *N. macfarlanei*, this could happen because the pollen is carried in large clumps. The tendency, however, of this species to form large clumps of pollen grains suggests that they are less likely to be carried by wind. Further, it would seem unlikely that a plant which produces so many visitation rewards would also depend on wind for pollination and, indeed, many females, at least at the Mt. Purun plot, flowered at a height less than 2 metres, a height which is generally considered to be below the sub-canopy strata of an upper montane forest. This stratum is often densely occupied by shrubs and treelets and wind movement is much more restricted. It is true that the eventual exposure of the inflorescences from out of the canopy would facilitate wind pollination but it is clear that there are many inflorescences that will never

reach that height. Based on the above deliberations, it is concluded that this species is insect-pollinated.

N. macfarlanei has a simple flower structure. Faegri & van der Pijl (1979) and Bawa *et al.* (1985) have suggested that flowers which are small, pale and which lack the morphological specialisation that restricts accessibility to their rewards by a limited range of insects, are more likely to be associated with varied assemblages of relatively small insects such as bees, flies, wasps and beetles. This would seem to apply to this species since, apart from the simple flower structure and the accessibility of its rewards, the diverse range of invertebrates that were observed to visit the flowers suggests that the flower is not specialised. The simple floral morphology, coupled with the synchronous maturation in the female inflorescence seem to be strategic sexual adaptations to the habitat. The low observed frequency of pollinator visits in the upper montane forest would lead to a higher degree of competition for pollinators (Frankie *et al.*, 1974; Wheelwright, 1985; Rathcke & Lacey, 1985). Such pressures may have selected these reproductive attributes so as to enhance the chances of successful pollination and in this context, Tanner (1982) and Sobrevila & Arroyo (1982) have noted that pollination systems involving small generalist insects appear to be more widespread in the tropical cloud forests.

Although the Muscidae flies recorded the highest frequency of visitation to the flowers and pollen grains were observed on their bodies (Appendix 23), it is difficult to conclude that they are the main pollinators because they did not systematically 'work' the male and female flowers. Ants were the second most frequent insect visitors to the flowers and, although they have not been recognised as legitimate pollinators (Proctor & Yeo, 1973; Faegri & van der Pijl, 1979), it would be worthwhile to examine the possibility that the flowers may also receive some forms of benefits from their visits.

The more frequent visits by the more short-tongued insects compared to almost none of the long-tongued species (*e.g.* Lepidoptera) is perhaps not surprising in view of the exposed nature of the plants' floral rewards which do not require much probing (Proctor & Yeo, 1973). Willis and Burkill's (1895-1908) work on the plant-pollinator relationships in Great Britain showed that the less specialised flowers most visited by short-tongued Diptera are also those that were most visited by insects in general. Free (1960), Frankie *et al.* (1976) and Bawa (1977) have shown that many of the small diverse insects have restricted foraging ranges and that they often return to the same resource patch and it has been concluded that the temporal and spatial variations in the quality and quantity of floral rewards may promote movement of these insects between patches (Bawa, 1980). In the case of *N. macfarlanei*, the resource patches are not likely to be large because of the synchronous anthesis within the female inflorescence and the close proximity of flowers, thus an insect may forage with ease. In any case, the patch will most probably not exceed the maximum distance of the nearest male neighbour (range 1-34.7 metres, mean distance 15.1 ± 11.02 metres; see 3.3.2 for results). An important consequence of this is that long distance gene flow through pollen and introgression is not likely to happen between populations placed far apart. This is particularly relevant to populations at isolated summits and summits in a large massif and, as a consequence, new genotypes will arise only as a result of genetic, evolutionary and stochastic forces acting on a specific population at a particular locality. If these insects are indeed the legitimate pollinators for *Nepenthes* spp., then this could explain why natural hybrids have mostly been found close to parent populations.

4.4.4 Fruit production

Despite having 100% of female flowers in the population reaching receptivity, only 59.2 % of these had successful fruit set while the remaining 40.8% aborted. Abortion is considered

to be a general phenomenon (Lloyd, 1980; Lloyd *et al.*, 1980; Stephenson, 1981; Bawa & Webb, 1984) and may occur either at the stage of pre-anthesis or post-fertilisation. The abortion at the former stage may occur as early as small bud stage while the abortion at the latter stage may occur soon after flower maturity. Although the percentage of abortion was greater in females than in males, there is much variation between female plants. The variation in flower development is the result of both intrinsic and extrinsic factors including the selection for increased pollen dispersal (Bawa & Webb, 1984), the availability of pollinators (Schemske, 1977; Bierzychudek, 1981; Rathcke, 1983) or resources (Abrahamson & Gadgil, 1973; Newell & Tramer, 1978; Lloyd, 1980) and the interspecific gene flow (Levin & Anderson, 1970; Waser, 1978). The abortion at different floral stages indicate that plants differ in the relative use of maternal investments. Lloyd *et al.* (1980) have demonstrated that the greater variability occurred in fruit maturation than in ovary development. The post-fertilisation abortion of fruits may be regarded as a mechanism to regulate the genetic quality of the offspring (Lloyd, 1980).

This lack in reproductive efficiency is made somewhat less critical by the ability of a fruit to produce many seeds even when the seed set is not high. The gross estimate of the number of mature seeds produced by the sample population during the phenological period is extremely high (see 4.3.4). Although no attempts were made to record the seed set of all plants in the sample population during that period, the researcher was able to collect more than 100,000 seeds that was required for the storage and micropropagation studies. The observations of large number of seeds in *N. macfarlanei* in this study were similar to the observations of Danser (1928) and Phillips & Lamb (1978) on unspecified species but different from the observations of Kaul (1982) with *N. lowii*.

4.4.5 Flowering in *Nepenthes* sp.

Brief observations on two other species occurring at Mt. Purun (*N. gracillima* and *N. sanguinea*) showed that while flowering in *N. gracillima* is aseasonal and sporadic, *N. sanguinea* did not flower or fruit at all. Many of the *N. sanguinea* plants were juveniles. The flowering behaviour and floral syndrome of *N. gracillima* resemble those of *N. macfarlanei* (personal observations). Lim & Prakash (1973), Kato (1993) and Kaul (1982) have described similar behaviour and syndromes for *N. gracilis* (lowland species), *N. lowii* and *N.villosa* (montane species) respectively. It is therefore concluded that the mode of pollination in *Nepenthes* spp. in general is likely to be predominantly entomophilous.

4.5 CONCLUSION

In relation to conservation, the reproductive attributes of *N. macfarlanei* seem to be in accordance with what would appear to be the requirements for maintaining population survival and viability in its natural habitats. The strictly allogamous breeding system of *N. macfarlanei* results in outcrossing and creates opportunities for intra- and interspecific gene flow to occur in and between populations. High levels of heterozygosity and genetic variability will be maintained in its populations and this will buffer the detrimental effects of selection pressures on the genetic composition of its population. Even though to a large extent, a high degree of heterozygosity in the genetic structure of its population will favour survival rather than extinction, there are other important factors that will influence survival rates. These include the breeding capacities of the populations and plant-pollinator-environment interactions. Although this species is capable of producing large amounts of propagules during its lifespan, natural selection will select for those that are reproductively fit, resulting in a significantly lower number of mature individuals in the population. The dependence of *N. macfarlanei* on insects for pollination indicates that interactions, either intrinsic or extrinsic, which directly affect the survival of the insect population at the same time affect the survival of this species. These interactions are in turn intricately linked to the variation within the habitat where the insect species and *N. macfarlanei* occupy. In cases where the variations are the consequences of man's activities in the surrounding habitat, measures must be taken to ensure that sufficiently large numbers of both potential pollinators and *N. macfarlanei* are conserved

CHAPTER 5

LONG-TERM STORAGE AND VIABILITY OF SEEDS OF *N. macfarlanei*

LONG-TERM STORAGE AND VIABILITY OF SEEDS OF *N. macfarlanei*

5.1 INTRODUCTION

5.1.1 The Aim

Storage of genetic resources via seeds or other genetically-stable propagules has long been recognised as important means of *ex situ* conservation because samples of genetic diversity can be maintained in a secure manner. This approach, utilising the techniques of conventional storage and cryopreservation, is widely practised, particularly in the germplasm conservation of agricultural crops. Many of such crops have 'orthodox' seeds *i.e.* seeds which can be desiccated to a low moisture content and tolerate low storage temperatures (Roberts, 1973). In contrast, such techniques have not been entirely successful with 'recalcitrant' seeds due to their inabilities to be desiccated below a critical moisture content and to withstand freezing temperatures. These seeds lose viability once they are dried to a moisture content below a relatively high critical value. Use of low temperatures and cryopreservation during storage have vast potential for many species because they reduce, to a great extent, seed metabolic processes and in the case of cryopreservation places seeds in suspended animation for an indefinite period. For the purpose of conservation, this study aims to determine the physiological nature of *N. macfarlanei* seeds and to assess whether conventional storage and cryopreservation can be applied with reasonable success to *N. macfarlanei.*

5.1.2 Literature review

Past studies suggest that the seeds of *Nepenthes* spp. may have limited viability (Garrard, 1955; Lim & Prakash, 1973; Slack, 1979). Lim & Prakash (1973) reported a very low germination percentage in *N. gracilis* seeds after one week in storage at 30°C, indicating that deterioration had occurred at this temperature. In contrast, Corker (1986) reported

that seeds of *N. mirabilis*, stored for two months at 25°C, gave the highest germination percentage among treatments. She observed that, in general, stored seeds had a higher germination percentage compared to those without storage. Freshly collected seeds showed no germination while seeds stored for 48 hours, 1 week, 3 weeks and 1 month had germination percentages of 8%, 24%, 42% and 54% respectively; there was, however, a significant decline after 3 and 4 months in storage. In an attempt to describe the contrast behaviour of *N. mirabilis* seeds from *N. gracilis*, she suggested that the increase in the germination percentage of *N. mirabilis* seeds with longer storage periods was an adaptive feature of the species related to its survival in the wild. *N. mirabilis* is the only species whose range extends into the subtropical climate region where marked seasonal changes in temperature, humidity and rainfall occur. At least in Hong Kong, the species produces seeds between July and September but not all are released at the same time. Those that are released at the end of autumn will need at least three months of dormancy to overcome the unfavourable winter climate. This suggestion is however rather vague as her results showed that the germination percentage of seeds declined significantly after two months in storage.

Corker *loc.cit.* showed that the time for *N. mirabilis* seeds to germinate varied from 31 to more than 120 days. Seeds that were stored for one month had the lowest time (31 days) while seeds that were stored for four months took more than 120 days to germinate. If it is permissible to consider merely absolute values in seed studies, this result could indicate that either seed deterioration had taken place more rapidly after two months in storage or that secondary dormancy had been induced during storage. Also, Green (1967) noted that seeds of *N. gracilis* that were stored require a longer period for germination.

These seed viability results for *N. gracilis* and *N. mirabilis* should be interpreted with caution because both studies did not provide statistical results and therefore, the deductions are not fully convincing. Even if the results may be taken at face value, other factors exist. First, only a single batch of seeds developing from one flowering season was used. Viability in *N. mirabilis* seeds is likely to be affected by the different climatic conditions experienced throughout its range while in *N. gracilis,* its viability may be slightly more restricted as a result of its more tropical distribution. Second, the experimental designs and procedures used were not according to guidelines set by the International Seed Testing Association (1931-1985) (Grabe, 1987). Third, studies with seeds of other species, either orthodox or recalcitrant, have shown that high storage temperatures are likely to encourage continuous seed development and fungal infections, thus reducing seed longevity and viability (Roberts, 1973; Stanwood & Bass, 1981; Leopold & Vertucci, 1986). The high temperature experimented by Lim & Prakash *loc. cit.* (30°C) and Corker *loc. cit.* (25 °C) is likely to have similar effects on *N. macfarlanei*. From the above arguments, it is likely that the loss of viability after storage could well be the outcome of inappropriate methodology rather than the inherent lack of viability. Although the species are tropical in nature, the seed size resembles that of orthodox seeds, and if the above results arise basically from inappropriate methodology, it is possible that seeds of *Nepenthes* spp. are actually orthodox.

5.2 MATERIALS AND METHODS

5.2.1 Experimental procedures

5.2.1.1 Seed procurement and selection

Mature seeds were used as experimental material and these were obtained from GH. Seed sorting was done in the Herbarium and the Seed Technology Laboratory, Environmental Science, FRIM, Kuala Lumpur. The mean daily temperatures and relative humidities for the two locations were 23.3°C & 69.8% and 22 °C & 66.1% respectively. As a large number of seeds were required in this study, maturing infructescences were bagged with very fine nylon mesh to prevent seed loss. Seed batches varied in collection dates. No specific selection was done on mature fruits; all that were available were used. In all the following experiments, seeds were randomly mixed prior to counting.

5.2.1.2 Calculation of moisture content (mc)

The procedure used to determine the percentage mc of each working sample followed ISTA (International Seed Testing Association) 1993 guidelines. Unless specified, three hundred seeds, divided into three replicates were used to determine the mc in the following experiments. Initial fresh weights of replicates were obtained after which they were oven-dried at 103° C for 17 hours. Upon removal from the oven, they were placed in a desiccator to cool. Dried weights were subsequently obtained. The calculation for the percentage mc in each replicate is as follows:

$$\text{Initial mc (\% of fresh weight)} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100$$

When it was required to desiccate the replicate before storage, the desired mc is calculated in two steps; the first step involved the calculation of the percentage loss of mc during desiccation. This was calculated using the above formula in which the dry weight was

substituted with desiccated weight. The second step involved the subtraction of the loss from the initial mc. The initial mc is calculated from the result obtained from the oven-drying procedure.

5.2.1.3 Germination of seeds

In all experiments, 400 seeds, divided into four replicates were used (ISTA, 1993). Seeds were planted onto germination trays containing moist tissue paper floated on water. There were four trays. Replicates for all treatments were placed in a germination chamber with a mean temperature of 23.3°C and PAR of 12 to15 $\mu Em^{-2}s^{-1}$. Seeds were observed daily for germination and recording began on the day the first seed germinated, terminating at day 80. Germination was considered to have taken place when the radicle had protruded 1 mm from the seed coat. The percentage of normal developing seedlings was also determined for each replicate at the end of the observation period. Time (G) taken for the replicate to attain 50% germination was calculated as follows :

$$G = \frac{\Sigma (Dn)}{\Sigma N}$$

where

n = number of seeds which germinated up to day D

D = the number of days, counted from the beginning of test, taken by the replicate to achieve at least 50% germination

N = total number of seeds in the replicate

For replicates that did not provide the exact value of 50% germination, the time was calculated based on the day taken by that replicate to attain a value greater and closest to 50%. In all experiments related to storage, viability is expressed as a combination of germination percentage and germination time.

5.2.1.4 Packaging of seeds

Seed lots were packed in envelopes made of household-grade aluminium foil of 6 x 6 cm size. Envelopes were prepared by double-turning the three sides of the foil. Packages that were designated for conventional storage were placed in polythene bag and heat-sealed while those that were designated for storage in liquid nitrogen were wrapped again in aluminium foil and attached to cryo-sticks with Parafilm.

5.2.1.5 Statistical analysis

All data were analysed with ANOVA according to the experimental designs. Germination percentages were arc-sine transformed before analysis (Sokal & Rohlf, 1981); means reported were back-transformed. The software used to calculate ANOVA was CSS (Complete Statistical Systems, version 3.1, developed by Statsoft Inc.). LSD (least significant difference) and T-method tests were used to compare treatment means. Graphs were generated using Microsoft Excel version 5.0.

5.2.2 Determination of the appropriate composition of a seed batch for storage

To determine the appropriate seed batch for storage experiments, two different batches were used. The first batch consisted of seeds from one plant and the second batch consisted of seeds from different plants.

In the first batch, 2000 seeds, randomly removed from one infructescence, were divided into twenty replicates. In the second batch, 2000 seeds, randomly removed from many plants, were divided into twenty replicates. This seed number was chosen as this was the number used for the mc test in storage experiments. Initial mc was determined for both batches. Results from this experiment showed that the mc of both seed batches were not

significantly different. Therefore, in subsequent experiments, the seed batch which is derived from many plants was used.

5.2.3 Determination of the mc curve

In this experiment, two desiccation chambers were used; they were a laminar-flow cabinet and a desiccator containing silica gel. Specific periods of desiccation were used in each of the following experiments.

5.2.3.1 Desiccation by laminar airflow

Seeds were exposed to the laminar airflow for periods up to 7 hours and samples were removed at intervals of one hour. Prior to desiccation, the cabinet was allowed to run for two hours to enable the airflow to stabilize at an average velocity of 0.46 m/s. 3000 seeds, divided into three replicates were used for each treatment. Seeds were evenly distributed on an aluminium foil and placed in the cabinet. At each hour interval beginning with 0, 3 replicates were removed from the cabinet. The mc of desiccated replicates was determined and the means plotted against a period of 7 hours. Apart from a thermohydrograph (see below), no other apparatus that could affect the rate of desiccation was placed in the laminar cabinet. As it was not possible to obtain the number of seeds required for this experiment in one collection batch (24,000), several batches were used. However, to ensure that the initial mc of these batches were not contributing factors, they were subjected to an ANOVA analysis. Batches that gave significantly different mean initial mc were discarded. This test was repeated once. Results reported is the mean of two experiments.

The laminar cabinet used was HLF Gelman Series (Gelman Science Pty. Ltd., Australia). During the drying period, a thermohydrograph (Sato, Sato Keiryoki MFG, Japan) was placed in the chamber to monitor the fluctuations in temperature and relative humidity.

5.2.3.2 Desiccation by silica gel

Seeds, evenly spread out on an aluminium foil, were suspended 10 cm above a 3-cm layer of newly-dried silica gel in a 4-L desiccator at 23°C. Due to the lack of seeds at that time, the drying period was reduced to 6 hours and replicates were sampled at two hourly interval beginning with 0. 3000 seeds, divided into three replicates, were used for each treatment. The mc of desiccated replicates was determined and the means plotted against a period of 6 hours.

Results from this experiment (and experiment 5.2.3.1) showed that the rate of mc loss was greatest during the first two hours of desiccation. Therefore, another test was conducted with sampling done at 0, 0.5, 1.0, 1.5 and 2 hours using 3000 seeds, divided into three replicates. Once again, to ensure that the initial mc of these batches were not contributing factors, they were subjected to an ANOVA analysis. Batches that gave significantly different mean initial mc were discarded. This test was repeated twice.

5.2.4 Storage

5.2.4.1 Conventional storage

Despite repeated tests, the rate of mc loss in desiccated replicates were erratic when laminar airflow was used as the drying agent. This was probably due to the high relative humidity of the surrounding air in the cabinet, created by the forest close to the Environmental Science building. As a result, drying with silica gel was selected for this experiment.

Due to the difference in the initial mc, it was difficult to obtain within tolerance limits, similar mc levels for replicates designated for 2 and 4 months storage. Therefore, hours of desiccation was used instead as a guide. For each treatment, replicates were removed and calculated for mc. This meant that the mc levels vary between treatments. After each hour, 400 seeds were removed from the desiccator, sealed in aluminium foil (Stanwood & Bass, 1981) and labeled. A separate lot of 300 seeds, divided into three replicates, were also removed, sealed and labeled. These envelopes were weighed separately to obtain the mc after desiccation. These packages were placed into plastic bags, sealed and stored at the required temperature. Temperatures used were room temperature (23°C), 4°C and -18°C and the duration was 2 and 4 months. After storage, the 400 seed lot was germinated while the other three replicates were processed for mc; germination was monitored over a period of 80 days. Final germination percentage and time taken to achieve 50% germination were determined. One control set, containing a lot of 400 seeds and 3 lots of 100 seeds each was processed for germination and mc respectively at the beginning of the experiment.

5.2.4.2 Cryopreservation

Because of the lack of seeds, a single period of desiccation (2 hours) prior to the attempted cryopreservation was investigated along with a non-desiccated control sample. One batch, consisting of 400- and 300-seed lots (the latter were divided into three replicate 100-seed lots) were desiccated for 2 hours, weighed, sealed and stored at -196°C (liquid nitrogen) for 2 months. For the control, one further batch of 400- and 300-seed lots was treated in the same manner except that the desiccation stage was excluded. After recovery from liquid nitrogen, the 400-seed lots were tested for germination while the other replicates were processed for mc determination; germination was monitored over a period of 80 days and the final germination percentages and mean germination times were determined.

5.3 RESULTS

5.3.1 Determination of the appropriate composition of a seed batch for storage

ANOVA results showed that the mean initial mc's of the two seed batches described (see 5.2.2) were not significantly different ($P>0.05$, $n=20$) (Appendix 25). Data from these batches showed that the mc of fresh seed upon release from the fruit varied between 7.2% and 12.6%. However, the differences in means for seed batches collected at different times of the year were sometimes significant (Table 5); unfortunately this could not be correlated with the climatic variation as the data on the climatic conditions at GH were very basic and incomplete. The fluctuation in the initial mean mc is perhaps not surprising, since the seed batches were heterogeneous and, in addition, the variation in the environment prior to collection has been known to influence the mc of seeds (Roberts & Ellis, 1984). This large variation in the initial mc, however, creates certain complications, including the difficulty in obtaining a mc curve that is appropriate for different seed batches. Since the mc curve obtained from the desiccation process, particularly in the early stages of desiccation, is likely to differ between seed batches collected at different dates, relatively large samples of seeds from each seed batch must be destroyed to obtain the reference curve. For *N. macfarlanei* in particular and *Nepenthes* spp. in general, the use of a relatively large sample of seeds for mc determination (at least 100 seeds per lot) is also necessary as the seed is minuscule in weight and, unless a sophisticated weighing balance can be made available, the use of small sample sizes would increase the measuring error. In cases, therefore, where the breeding population is small and the production of viable seeds is relatively low or when programmes are undertaken to rescue threatened populations, the destruction of large samples of seeds in such a manner could considerably reduce the value of long-term storage as an approach to conservation.

Table 5 : A matrix showing a comparison of the mc's of seed batches collected at different dates

collection date	mean initial mc (%)	collection date							
		22/7/93	12/8/93	7/10/93	2/9/94	6/12/94	13/12/94	8/2/95	16/2/95
22/7/93	15.4 ± 1.86		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
12/8/93	11.1 ± 0.53	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
7/10/93	8.8 ± 0.32	**	n.s.		n.s.	n.s.	n.s.	n.s.	n.s.
2/9/94	9.4 ± 0.29	**	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.
6/12/94	12.1 ± 0.13	n.s.	n.s.	n.s.	n.s.		**	n.s.	n.s.
13/12/94	9.8 ± 0.16	n.s.	n.s.	n.s.	n.s.	n.s.		**	
8/2/95	11.9 ± 0.51	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.
16/2/95	11.3 ± 0.16	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	

The initial mc measured in the seeds of *N. macfarlanei* was relatively low (4.6% to 15.4%; see Table 6 for the former value and Table 5 for the latter value) and was comparable to that of orthodox seeds (Chin *et al.*, 1989; Ellis *et al.*, 1989; Ellis *et al.*, 1990). In such seeds, drying occurs when the seed is mature; this process, called maturation drying, is an integral process occurring only at the mature phase of development while the fruit is still attached to the parent plant (Bewley & Black, 1985; Kermode & Bewley, 1988; Chin *et al.*, *loc. cit.*). It was difficult to determine whether seeds of *N. macfarlanei* had undergone such drying because no tests were conducted on the germination and mc of seeds at various stages of maturity.

In addition, Chin *et al. loc. cit.* have noted that the sizes and weights of orthodox seeds are relatively small in comparison with those of recalcitrant seeds. In *N. macfarlanei*, the mean seed length without the filiform seed coat was 2.9 ± 0.41 mm (n=20), the mean diameter was 1.0 ± 0.16 mm and the mean fresh weight of a seed was (1.67 ± 0.108) x 10^{-4} grams. These features of *N. macfarlanei* seem to fit the description of an orthodox seed.

5.3.2 Determination of the mc curve

5.3.2.1 Desiccation by laminar airflow

The changes in the mean mc of replicates desiccated over a period of 7 hours is given in Fig. 16. There was hardly any significant loss in the mean mc over the first two hours of desiccation, but a decrease occurred after 3 and 4 hours, but thereafter the mean mc increased. This increase in the mean mc after 4 hours could have been the result of sampling errors arising from the variation in initial mean mc between the seed batches. The effect of such variation, however, should have been minimized by the randomization process that was adopted. A more likely explanation was that the RH of the surrounding air, which fluctuated between 60% and 77% during the experiment, had an influence on the mean desiccated mc. King & Roberts (1980) have shown that small seeds with permeable seedcoats are more likely to be affected by minor fluctuations in relative humidities compared with large seeds with impermeable seedcoats. Seeds of *N. macfarlanei* are likely to follow this pattern because of their small size (see section 5.3.1) and the morphology of their seedcoats. It was concluded, therefore, that this method of desiccation is not effective with this species and it was discarded in favour of desiccation by silica gel.

5.3.2.2 Desiccation by silica gel

The changes in the mean mc of replicates desiccated in the presence of silica gel over a period of 6 hours are given in Fig. 17. With this technique, the loss in mc was greatest in the first two hours of desiccation, and Fig. 18 shows the changes in the mean mc over an initial period of two hours for the two separate tests. The curves obtained here were different from the one obtained by using laminar airflow, leading to two conclusions. First, the RH of the surrounding air probably did affect the rate of mc loss during desiccation in the laminar airflow and, in consequence, the seed mc could not be reduced to significantly

Fig. 16 : The changes in the mean mc of seed lots desiccated over a period of seven hours by laminar airflow

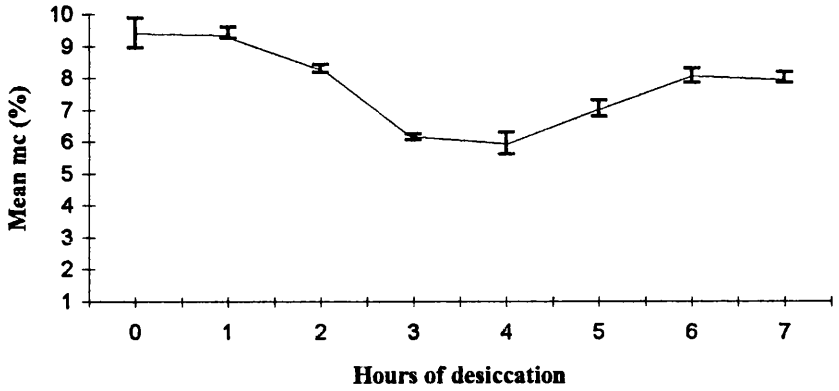


Fig. 17 : The changes in the mean mc of seed lots desiccated over a period of 6 hours in the presence of silica gel (test 1 *)

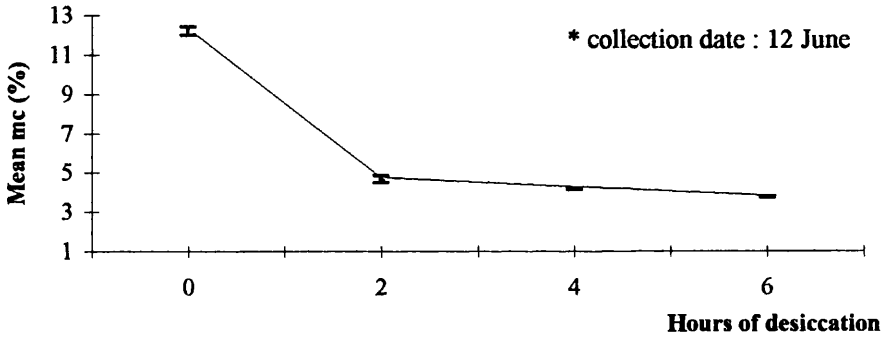
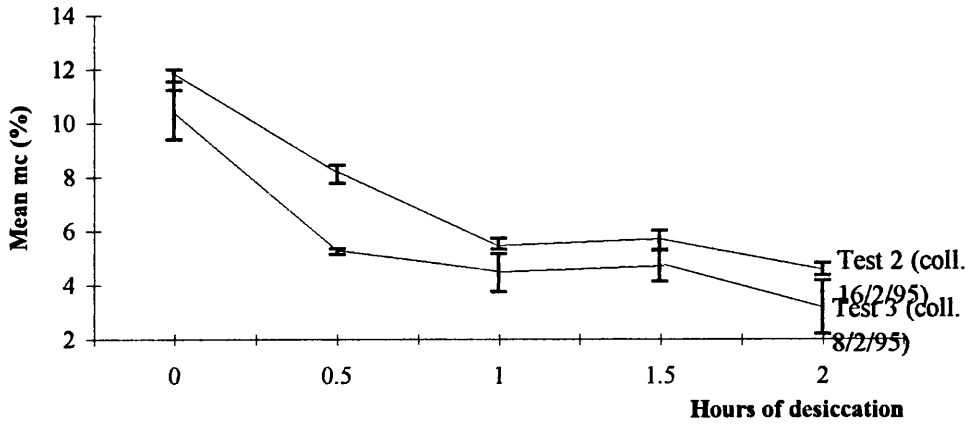


Fig. 18 : The changes in the mean mc of seed lots desiccated over a period of 2 hours in the presence of silica gel for two separate tests



lower levels. The desiccation test with silica gel clearly showed that *N. macfarlanei* seeds can be desiccated to levels comparable with those reported for orthodox seeds (IBPGR, 1985a), although at this point, it was not known whether the viability of seeds could be maintained under these conditions. Second, the dissimilarities between the results obtained with the two drying approaches showed that the correct choice of desiccation procedure is important with the seed biology of the species of interest being crucial. In species with large-sized, recalcitrant seeds, drying by laminar airflow is a fairly new technique (Krishnapillay, 1987; Chin *et al.*, 1988; Pence, 1990; Normah & Vengadasalam, 1992; Marzalina, 1995) while in the desiccation of orthodox seeds, silica gel is commonly used. In the case of *N. macfarlanei* (and probably other *Nepenthes* spp.), the more reliable approach is likely to be the one employing silica gel as the drying agent.

5.3.3 Storage and viability

Table 6 provides a summary of the germination percentages and mean germination times obtained for seed batches in each storage treatment. Analysis of the results shows that the mean cumulative germination percentage at the final day of observation and the mean germination time at 50% germination were significantly different between treatments ($P<0.05$, $n=4$) (Appendices 26 & 27). Control (no desiccation and storage, treatment S) gave the highest mean germination percentage compared to all other treatments ($86 \pm 0.2\%$, Fig. 19), although its mean germination time was not significantly different from the seed batches of some treatments (Table 6, Fig. 20). Apart from treatment N (with lowest germination percentage), the mean germination percentages for seed batches stored for two months and for four months were not significantly different from each other, despite the use of different levels

of mc, storage temperature and duration, indicating that none of the treatments was particularly beneficial. However, preliminary results from a less complete experiment with seed batches stored over a period of 12 months (results not reported here due to the meagre data available) showed that there was no germination of fresh seeds stored at -18°C. Fresh seeds that were stored at 4°C for the same period gave 54% germination compared with the 61.5% produced by an equivalent seed batch stored at 4° C for four months (treatment B; Table 6). Similarly, seeds desiccated for 1 hour and stored at 4°C for 12 months gave only 49% germination compared with the 75% produced by an equivalent seed batch stored at 4° C for four months (treatment D; Table 6). Although it is not known whether these percentages are significantly different from the percentages derived from experiments of 5.2.4.1, the reduction in germination does seem to indicate a certain decline in viability. The rate of decline with longer storage period may be reduced considerably by the use of low temperature, such as that provided by cryopreservation, since at this temperatures, all metabolic processes in the seeds are suspended.

Table 6 shows that while seed batches of *N. macfarlanei* could be desiccated to very low levels of mc, these batches are also capable of absorbing moisture during storage; most batches, including those that were not desiccated, registered an increase in mc after storage and this phenomenon happened at all storage temperatures. However, the increase in the mc measured after storage was erratic and no trends could be linked either to the mc before storage or to the storage temperature. In addition, it could not indicate whether the level of mc is affected by the duration of storage. This behaviour is possibly the result of a combination of experimental errors and the intrinsic properties of the seeds. The experimental errors that could have taken place at several stages during the experimental procedure include:

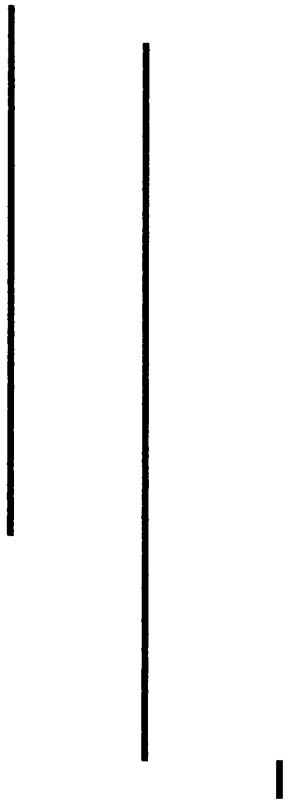
Table 6 : Mean mc (%), germination percentage and mean germination time for seed batches stored under a range of conditions

Temp (°C)	Des (hour)	Storage period (months)																		
		0					2							4						
		coll. date	trt. code	initial mc (%)	final G (%)	mean G time (days)*	coll. date	trt. code	mc (%) initial	mc (%) before st	mc (%) after st	final G (%)	mean G time (days)*	coll. date	trt. code	mc (%) initial	mc (%) before st	mc (%) after st	final G (%)	mean G time (days)*
control	0	10/2/95	S	10.07	86	17.33	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	0	-	-	-	-	-	10/2/95	G	4.77	4.77	7.76	64.25	20.23	19/1/95	H	11.97	-	10.1	57	35.63
	1	-	-	-	-	-	10/2/95	I	4.62	3.28	5.38	64.75	22.4	19/1/95	J	8.23	1.96	9.79	62	28.89
	2	-	-	-	-	-	17/2/95	K	6.19	3.35	4.85	55.75	23.13	19/1/95	L	5.94	8.53	7.62	57.5	31.61
4	0	-	-	-	-	-	10/2/95	A	6.36	6.36	7.08	59.75	22.21	19/1/95	B	11.81	-	8.18	61.5	34.57
	1	-	-	-	-	-	10/2/95	C	4.34	2.04	6.98	70.5	22.15	19/1/95	D	9.16	2.29	6.29	75	28.72
	2	-	-	-	-	-	17/2/95	E	5.48	1.93	2.64	59.75	20.43	19/1/95	F	5.18	7.79	4.58	57.75	32.71
-18	0	-	-	-	-	-	10/2/95	M	4.95	4.95	8.77	71.25	23.68	19/1/95	N	10.91	-	14.75	46.25	36.92
	1	-	-	-	-	-	10/2/95	O	5	1.99	10.22	68.75	21.3	19/1/95	P	6.6	1.18	9.9	71.5	29.33
	2	-	-	-	-	-	17/2/95	Q	6.58	4.38	5.01	64.75	17.5	19/1/95	R	4.74	8.5	10.19	71.75	29.71
-196	0	-	-	-	-	-	17/2/95	T	7.73	7.73	18.07	66.5	18.3	-	-	-	-	-	-	-
	2	-	-	-	-	-	17/2/95	U	5.91	7.52	17.75	58.5	30.51	-	-	-	-	-	-	-

- = no experiment done
* = time taken to achieve at least 50% germination
Abbreviation : temp = temperature; des = desiccation; coll = collection; trt = treatment; st = storage; G = germination
number of seeds used in the mc test = 300, divided into three replicates
number of seeds used in the germination test = 400, divided into four replicates
Seeds germinated in 23.3 ± 1°C and 12-15 $\mu Em^{-2}s^{-1}$ PAR
Refer to Figures 19 and 20 for significance of mean germination percentages and mean germination times

Fig. 19 : Comparison of the mean germination percentage * for seed batches stored under a range of conditions (T-method using 95% comparison interval)

Ranking of the mean percentage	Ranking of treatment according to its mean germination percentage
lowest	N
	K
	H
	L
	F
	U
	A
	E
	B
	J
	G
	Q
	I
	T
	O
	C
	P
	M
	R
	D
highest	S



NB : * percentages were transformed into arc-sine values

Description :
(1) Treatments which occurred on the same line have means which are not significantly different
(2) Treatments which did not occur on the same line have means which are significantly different

Fig. 20 : Comparison of the mean germination time * for seed batches stored under a range of conditions (T-method using 95% comparison interval)

Ranking of the mean time	Ranking of treatment according to its mean germination time
lowest	S
	Q
	T
	G
	E
	O
	C
	A
	I
	K
	M
	D
	J
	P
	R
	U
	L
	F
	B
	H
highest	N

NB : * time taken to achieve at least 50% germination

Description :
(1) Treatments which occurred on the same line have means which are not significantly different
(2) Treatments which did not occur on the same line have means which are significantly different

(1) Weighing. Because it was impossible to place a weighing balance in a desiccator, samples had to be removed from a relatively dry environment (in the desiccator) to a relatively humid environment (laboratory). Although the weighing balance was kept close to the desiccator and a beaker of silica gel was placed in the weighing chamber, the short exposure period during transfer could have induced an increase in mc.

(2) After removal from liquid nitrogen. Table 6 clearly showed that the greatest increase mc after storage occurred in seed batches that were cryopreserved. This increase was likely to have occurred during the thawing period which took place in the laboratory environment.

Although sealed aluminium foils were used to store seeds throughout the entire experiment, it is possible that the seals were not completely effective in excluding moisture. In that case, the increase in the mean mc after storage could be a result of the desiccated seeds of *N. macfarlanei* being able to re-absorb atmospheric moisture in the processing area and/or in the storage chamber. Studies with other plant species, at least those bearing orthodox seeds, have shown that the presence of a high mc during storage is detrimental (Roberts, 1960; Harrison & Carpentar, 1977; Ishikawa & Sakai, 1978; Ellis & Roberts, 1980; Tompsett, 1987; Chin *et al.*, 1987; Pence, 1990; Normah & Vengadasalam, 1992). In addition, some studies have reported damage and loss in viability when desiccated seeds/embryos are rehydrated, particularly when the uptake of water occurred rapidly (Hobbs & Obendorf, 1972; Powell & Matthews, 1978; Duke & Kakefuda, 1981; Vertucci & Leopold, 1984; Ellis *et al.*, 1990). The ability of *N. macfarlanei* seeds to be desiccated to very low mc and their susceptibility to rapid re-absorption of moisture from the atmosphere during and/or after storage could adversely affect the seed tissues and this may be the major causes leading to the decline in viability. However, there could be another factor that may have contributed to the observed decline in viability

during storage; this was the induction of secondary dormancy during storage. Low temperature treatment has been known to trigger physiological changes in seeds of some plant species thereby resulting in the decrease in responsiveness towards germination stimulus (Ikuma & Thimann, 1964; Karssen 1970; Taylorson & Hendricks, 1973; Karssen, 1980); this could have happened in the case of *N. macfarlanei*, but its occurrence would not be fully supported by the above results. Although stored seeds gave poorer germination percentages compared with fresh seeds, germination nevertheless had occurred in all seed batches; with the exception of one batch (from treatment N), all batches produced more than 50% germination (Table 6). This relatively high germination percentage seemed to suggest that if unresponsiveness, which arises during dormancy, had occurred in a seed batch, it had occurred more by chance rather than by design. However, the possibility that dormancy had been induced during storage still exists; this was made evident by the significant increase in the mean germination time for seed batches that were stored at 4 months, compared with those that were stored at 2 months (Table 6, Fig. 20). The induction of dormancy-related behaviour is likely to create complications for the effective long-term storage of *N. macfarlanei* seeds.

Although the results of this study show that *N. macfarlanei* seeds could not be stored under the conditions investigated without significant loss of viability, they also show that the loss was not entirely drastic. However because of the decline, the most promising storage temperatures are likely to be very low temperatures, such as that provided by cryopreservation. Although, as required by the principles of orthodox seed storage, they may be desiccated to a low mc, it is apparent that moisture had been absorbed by the seeds, possibly during storage. The re-absorption of moisture is likely to be influenced by the morphology of the seed coat and the seed size. Therefore, future experiments would need to look into the application of more

reliable techniques to control the mc levels in stored seeds, including the use of suitable packaging materials.

5.4 DISCUSSION

The term orthodox seed refers to seeds that can be dried to low mc in which state they tolerate freezing temperatures without damage; in addition, their longevity is expected to increase in a predicted way with decreases in mc and temperature (Roberts, 1973). The relatively low mc in fresh seeds of *N. macfarlanei* (Table 6), the ability of the seeds to be dried to mc levels comparable to orthodox seeds (1.2 ± 0.5%) (Table 6) and their small size suggest that they are orthodox. Unfortunately, although they may satisfy the first requirement, they meet the second requirement only partially with regards to low temperature tolerance. The results of this study suggest that the seeds of *N. macfarlanei* are orthodox or semi-orthodox but until more research is done to ascertain the optimal interaction of mc and temperature on seed viability, it would be presumptous to classify the seeds of this species.

It is suspected that the large fluctuations in mc after desiccation and during storage could be the cause of the loss of seed viability in *N. macfarlanei*. The erratic viability response to a range of mc and temperature interactions possibly suggests that the effect of mc is not independent of storage temperature. Work on several orthodox species has shown that optimal mc varies with the storage temperature (Ellis *et al.*, 1989; Ellis *et al.*, 1990; Vertucci & Roos, 1993; Vertucci *et al.*, 1994) and, as a consequence, optimal drying protocols should, in effect, vary with temperature (Vertucci & Roos, 1993). This could perhaps apply to *N. macfarlanei*, but this can only be determined with more research. This mc complication is exacerbated by the variation in genotype and in conditions during ripening, harvesting, drying and processing (Roberts & Ellis, 1984).

Because of these complications, germplasm accessions of *N. macfarlanei* must be closely monitored. This is extremely crucial when higher temperatures are used as storage temperatures. The results showed that large fluctuations in seed mc will take place if processing procedures and working environment are not stringently controlled. Such facilities are often not available in national conservation programmes, and, although agricultural research institutions such as those of the CGIAR (Consultative Group on the International Agriculture Research) organization do have such facilities, their priorities are agricultural crops. The only option left is to attempt detailed studies in the area of critical and optimal mc at a range of temperatures. Although cryopreservation and/or very low temperature storage are promising because they reduce the costs of monitoring and regeneration, this should not limit research in higher temperatures. The use of higher storage temperatures, such as ambient temperatures, for conserving germplasm is appealing, particularly in tropical countries where infrastructure and facilities cannot be relied on. This is perhaps why at present the International Plant Genetic Resources Institute (IPGRI, formerly IBPGR : International Board for Plant Genetic Resources) is focusing on efforts to develop protocols that do not require low temperature storage (IBPGR, 1985b, 1992, 1993; Ellis *et al.*, 1993). In the case of the endemic *Nepenthes* spp., where the optimal mc has been achieved and the anticipated fluctuations arrested, it might be possible to utilize the naturally cool conditions found in the montane habitats as the storage environment, provided the temperature experienced by the habitat is appropriate and that the diurnal variations are within acceptance limits. In this way, very little infrastructure and facilities would be required to maintain a storage environment.

5.5 CONCLUSION

Due to the complications encountered in relation to the control of mc, it is not fully known whether the seeds of *N. macfarlanei* are orthodox or semi-orthodox. Their seed viability apparently declined during storage, but it is suspected that the fluctuation in the mc that probably occurred during storage played an important role. Although no significant progress was made in this study towards establishing a protocol for the conventional storage and cryopreservation of *N. macfarlanei* in particular and *Nepenthes* spp. in general, the results presented brings to awareness the limitations, created primarily by the intrinsic seed properties, that may occur with long-term storage. These limitations must be addressed with further research before any of the above strategies can be successfully applied for the *ex-situ* conservation of *N. macfarlanei*.

CHAPTER 6

MICROPROPAGATION AND PLANTLET REGENERATION OF *N. macfarlanei*

MICROPROPAGATION AND PLANTLET REGENERATION OF *N. macfarlanei*

6.1 INTRODUCTION

6.1.1 The aim

In vitro approaches, particularly those related to the multiplication of genetically-stable genotypes, are increasingly becoming an important tool in plant conservation programmes. In areas where habitats are threatened with destruction, it is one of the integrated strategies that can be employed to rescue plants and to maintain a small sample of the genetic diversity in the lost population. This approach is potentially useful also for the reintroduction of plants into natural habitats and/or botanic gardens or even for sale if this protects dwindling populations from over-collection. This approach could be particularly effective where rare species are concerned and when only a small quantity of material is available (Maunder, 1991; Rubluo *et al.*, 1993; Fay, 1994)

Micropropagation and regeneration have been attempted for several of the endangered *Nepenthes* spp. which occur mostly in the western part of the distribution range of the genus (Redwood & Bowling, 1990; Rathore *et al.*, 1991; Fay, 1994; Latha & Seeni, 1994). However, at the present time, no attempts have been made to use and modify these protocols for the endemic montane species in Peninsular Malaysia. This study aimed to produce a protocol for the mass-multiplication and plantlet regeneration of *N. macfarlanei.*

6.1.2 Literature review

In view of the horticultural importance of *Nepenthes* spp. and high degree of endemism within the genus, surprisingly little research effort has been given to their *in vivo* and *in vitro* propagation. Undoubtedly, this type of work has been undertaken by horticultural companies, judging by the number of cuttings available for sale but because of proprietary

rights such work will remain unpublished. To date, only one institution is actively involved in the micropropagation of a range of *Nepenthes* spp. This is the Royal Botanic Gardens (RBG), Kew, United Kingdom (Fay, 1994). In addition, several Indian researchers are working with *Nepenthes khasiana*, an endemic from Assam Hills (Rathore *et al.*, 1991; Latha & Seeni, 1994).

Much of the propagation work attempted at RBG is aimed towards enhancing collections in botanical gardens, reintroduction of species into their natural habitats and, where there are excess materials, provision for specialist growers. These aims are part of the integrated strategies for conserving genetic diversity (Falk, 1990). Over 90% of the *Nepenthes* spp. that have been investigated have been successfully propagated (Redwood and Bowling, 1990). Two species, *i.e. N. madagascariensis* and *N. carunculata* have flowered in culture, allowing the identification of male and female individuals for multiplication. Being a dioecious plant, knowing their sex prior to propagation for the purpose of reintroduction is important as this will influence the breeding capacity of the introduced population. Apart from *Nepenthes* spp., RBG is also actively undertaking micropropagation programmes for other rare or endangered species from the families of Orchidaceae, Droseraceae, Lentibulariaceae, Sarraceniaceae and representatives from several tree families (Fay, 1994). In addition to RBG, UNAM in Mexico is also currently attempting to develop *in vitro* protocols for the recovery of endangered orchid and cacti (Rubluo *et al.*, 1993).

N. khasiana has been successfully established from nodal stem segments of seedlings (Rathore *et al.*, 1991; Latha & Seeni, 1994). In the study conducted by Rathore *loc. cit.*, between ten and twelve shoots have been produced from nodal shoot segments of 1.0-1.5 cm length on a modified Murashige and Skoog (MS) medium containing combinations of plant growth hormones; 80% of these shoots rooted on MS medium provided with NAA

and kinetin. The multiplication medium used contained a complex mixture of phytohormones, protein derivatives and anti-oxidants. Unfortunately, there was no report of a comparison between treatments with the complex supplements and a basal medium control. The apparent dependence of *N. khasiana* on a complex array of exogenous phytohormones for proliferation is refuted by the work of Latha & Seeni (1994) who reported that proliferation of shoots had occurred on nodal stem segments cultured in Woody Plant Medium supplemented with BA as the sole phytohormone.

6.2 MATERIALS AND METHODS

6.2.1 Experimental procedures

6.2.1.1 Seed source

The following experiments used mature seeds as parent materials for initiating cultures. A large proportion of the seeds was obtained from GH while a smaller proportion was obtained from CH. Seed batches were collected at different times of the year and from different plants. In all batches, only fully developed seeds were used; unless specified, immature seeds were discarded. All seeds were processed in the Herbarium and Seed Technology Laboratory at FRIM, Kuala Lumpur. Where necessary, seeds were stored at 4°C prior to use. Wild plants were not used as parent materials as their removal from the habitat would reduce the population number and consequently, its breeding capacity.

6.2.1.2 Culture vessels

In all experiments, borosilicate reagent bottles, Pyrex wide-mouthed conical flasks (100 and 250 ml), Magenta vessels (3x3x3 inches) (Magenta Corporation, Chicago) and locally-packed, gamma-irradiated disposable petri dishes (Labchem Sdn. Bhd., Petaling Jaya, Malaysia) were used. All new glassware was first soaked overnight in 1% hydrochloric acid. A standard procedure of cleaning for all glassware was then followed: after a preliminary soak in tap-water containing "7-X" detergent (Flow Laboratories Australasia Pty. Ltd., N.S.W. Australia) the glassware was rinsed three or four times in running water, followed by a rinse in single distilled water before drying.

6.2.1.3 Chemicals

The chemicals used in all experiments were of analar grade and purchased from Merck, Darmstadt, Germany and Sigma Chemical Company, Saint Louis, Missouri, USA. The

agar used for solidifying culture media was Agar Bacteriological Technical grade (Difco Laboratories, Detroit, Michigan, USA).

6.2.1.4 Preparation of stock solutions and culture media

The basal media used in the following experiments were Murashige and Skoog (1962) (Sigma M5519), Gamborg's B-5 Basal Medium with Minimal Organics (1968) (Sigma G5893), Nitsch & Nitsch (1969) (Sigma N5639), Schenk & Hildebrant (1972) (Sigma S6765), Vacin & Went (1949) (Sigma V5630). The inorganic salts and vitamins were prepared as four main stock solutions, namely macro elements (10x concentrated), micro elements (1000x concentrated), FeEDTA (10x concentrated) and vitamins (1000x concentrated). Sucrose ($30gl^{-1}$), myo-inositol (100 mgl^{-1}) and agar ($8gl^{-1}$) were added individually to each litre of basal medium prepared. At Bath University, U.K., the basal media used were in the form of prepared powders, obtained from Sigma Chemical Company.

The auxins, 2,4-D (Sigma D4517), 2,4,5-T (Sigma T2261), NAA (Sigma N0640), IAA (Sigma I2886), IBA (Sigma I5386) and cytokinins, BAP (Sigma B9395), kinetin (Sigma K3378) and 2-iP (Sigma D8532) were prepared as 10^{-6} M stocks which were frozen in 10ml aliquots for storage. These were defrosted when required during the preparation of medium.

The pH of all media was adjusted to 5.7 prior to addition of agar. The volume of media dispensed depended on the culture vessel used: 100 ml for conical flasks, 40 ml for Magenta vessels and 25 ml for petri dishes. Unless otherwise specified, the culture vessel used in the following experiments was petri dishes. Autoclaving was carried out at 121°C and 1.1 $kgcm^{-2}$ for 15 minutes after which the media were cooled and stored in a cold

room (7°C) until required. The media were allowed to warm to room temperature before they were used in culture work. Where petri dishes were used, the media were autoclaved in reagent bottles before being dispensed in a laminarflow cabinet.

6.2.1.5 Preparation of axenic seeds

Commercial bleach (Clorox, Colgate-Clorox (M) Ind., Petaling Jaya, Malaysia; the sodium hypochlorite concentration was not provided but it was most likely to be 0.525% w/v) (Msikita & Skirvin, 1989) was used as the surface sterilant. The concentration used was 3% (v/v) and the washing time was 20 mins; 30 ml of solution was mixed with a drop of Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma P1379). Seeds were vigorously shaken in the solution and then rinsed three times with autoclaved distilled water before culture. The entire process was conducted in the laminarflow cabinet.

6.2.1.6 Incubation of cultures

Unless specified, all cultures were maintained at 25 ± 1°C in a temperature-controlled room with a 12 hour photoperiod provided by Philips TLD 36W/54 fluorescent lights. The PAR ranged from 12 to 15 $\mu Em^{-2}s^{-1}$ and the relative humidity 67 to 75 %. At Bath University, U.K., the cultures were maintained at 28 ± 1°C, 16 hour photoperiod and 50 $\mu molm^{-2}s^{-1}$. Cultures that were incubated in the dark were wrapped with aluminum foil and placed in the same environment. The distribution of culture vessels on racks was completely randomized.

6.2.1.7 Statistical analysis

Where necessary, data were analyzed using analysis of variance (single classification) and T-method for unplanned comparison among treatment means. Percentages were transformed into arc-sine values while mean numbers were transformed into square-root

values before ANOVA was performed. Prior to square-root transformation, mean numbers were added with 0.5, as many explants did not produce buds. Only the results for significantly different treatments were back-transformed. Back-transformation was done according to the transformation used (Sokal & Rohlf, 1981).

6.3 RESULTS

6.3.1 Optimal germination medium

The basal media tested were Murashige and Skoog, Gamborg's B-5 Basal Medium with Minimal Organics, Nitsch & Nitsch, Schenk & Hildebrant, Vacin & Went together with 8 gl^{-1} agar + 30 gl^{-1} sucrose. No growth regulators were added. Eighty seeds were used for each treatment, divided between four petri dishes. They were monitored daily for signs of germination and the duration of observation was 11 weeks. Germination was recorded when the radicle protruded 1 mm from the seed coat. This experiment was repeated once.

Analysis of the results shows that the cumulative germination percentage at the end of 11 weeks varied significantly between the basal media used ($P<0.01$, $n=80$) (Appendix 28). Agar+sucrose medium gave the highest percentage (74.1 ± 5.78 s.d.) compared to the other basal media (Table 7). Media containing higher concentrations of macro and micronutrients seemed to inhibit germination.

Table 7 : The mean cumulative germination percentage for seeds sown in different basal media

Media	MS	GB5	NN	SH	VW	AS
Mean	38.4	47.4	50.4	41.2	47.6	74.1
s.d.	7.84	7.74	6.27	6.49	3.72	5.78

Incubated at 23-26° C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Number of seeds per treatment = eighty, divided into four replicates.
Duration of observation = 11 weeks

With the exception of the replicates in the agar+sucrose medium, most replicates in other media had not reached a mean germination time value of 50% by the end of the observation period (Appendix 29). The mean germination time for seeds sown on agar+sucrose medium was much greater (30.2 days ± 4.85 s.d.) compared with those germinated on

moist filter paper (17.3 days ± 1.76 s.d.). Factors influencing the extension of the germination time could include the intrinsic properties within the seed batches used, the effects of surface sterilization of the axenic seeds and the physical effects of the agar+sucrose medium. Despite the increase in germination time, agar+sucrose was clearly the superior germination medium; consequently, explants used in all the following experiments were derived from seedlings germinated on the agar+sucrose medium.

6.3.2 Micropropagation

6.3.2.1 To determine the optimal combination of medium and explant for the multiplication of shoot buds

In the following experiments, only BAP was selected as the cytokinin as this is the most commonly used cytokinin in tissue culture systems. Explants used in the following experiments included cotyledonary seedlings before the first leaf became visible, apical shoots and nodal segments. The control medium was full-strength basal MS supplemented with $30gl^{-1}$ sucrose.

6.3.2.1.1 Effects of BAP and basal media on the multiplication of shoot buds from cotyledonary seedlings

The experiment followed a completely randomized factorial design. The treatments were :

a. Basal Media : (all media supplemented with $8gl^{-1}$ agar & $30gl^{-1}$ sucrose)
   - Murashige and Skoog : Full-strength and Half-strength
   - Gamborg's B-5 Basal Medium with Minimal Organics : Full-strength and Half-strength
   - Nitsch & Nitsch
   - Schenk & Hildebrant
   - Vacin & Went
   - $8gl^{-1}$ Agar + $30gl^{-1}$ Sucrose

b. BAP :
   - 0 M
   - 10^{-6} M
   - $5x10^{-6}$ M
   - 10^{-5} M

Because of the difficulty in obtaining enough seedlings of the same age, seedlings from the same stage of development were used. All the cotyledonary seedlings selected were those that did not have a macroscopically visible shoot apex. As large numbers of seedlings were required for this experiment, only two replicates of five seedlings each were used for each treatment. The seedlings were evaluated after 30 days for the percentage of survival. For determining the mean number of shoot buds produced, seedlings were subcultured twice onto half-strength basal MS until the shoot buds became clearly defined and independent. As each subculture lasted one month, a total of 3 months was required to obtain the mean number of shoot buds.

Analysis of results shows that seedling survival varied significantly between treatments ($P<0.05$, $n=10$) (Appendix 30a). Agar+sucrose medium supplemented with $5x10^{-6}$ and 10^{-5} M BAP gave significantly higher survival percentages compared with other media (Table 8). There was no survival on the control medium (full-strength basal MS). Many of the treatment means had high standard deviations, perhaps as a result of the use of heterozygous genotypes.

In *N. macfarlanei*, the plumule of the mature embryo is reduced into a very small shoot apex, which is not macroscopically visible during germination. In media supplemented with BAP, small outgrowths proliferated around the shoot apex region within thirty days (Plate 16a). However, the percentage survival of such seedlings varied according to treatments (Table 8). In seedlings that survived, the outgrowths continued to grow in size until the entire region was covered with them. These outgrowths could be observed in this region, all of which were green and presumably photosynthetic. These outgrowths differentiated into shoot buds after several months of subculture in half-strength, basal MS

Table 8 : Survival and bud development from cotyledonary seedlings cultured on various basal media and BAP concentrations

Basal media	BAP (M)	Mean survival ± s.d. (%)	Mean number of shoots ± s.d.
Full-strength MS	0	0	0
	10^{-6}	0	0
	5x 10^{-6}	0	0
	10^{-5}	0	0
Half-strength MS	0	50 ± 2.0	1 ± 0
	10^{-6}	60.9 ± 8.65	1 ± 0
	5x 10^{-6}	60.9 ± 8.65	1.8 ± 0.23
	10^{-5}	40 ± 0	3 ± 1.41
Full-strength GB5	0	20 ± 2.5	1 ± 0
	10^{-6}	20 ± 10.4	0.5 ± 0.71
	5x 10^{-6}	40 ± 0	1 ± 0
	10^{-5}	11.3 ± 21.61	0.5 ± 0.71
Half-strength GB5	0	60.9 ± 8.65	1 ± 0
	10^{-6}	39.9 ± 0	1.5 ± 0.71
	5x 10^{-6}	60.9 ± 8.65	1 ± 0
	10^{-5}	11.3 ± 21.61	2.5 ± 3.53
NN	0	39.9 ± 0	1 ± 0
	10^{-6}	20 ± 2.5	1 ±0
	5x 10^{-6}	20 ± 10.4	0.5 ± 0.71
	10^{-5}	20 ± 0	1.5 ± 0.71
SH	0	40 ± 0	1 ± 0
	10^{-6}	60.9 ± 8.65	1 ± 0
	5x 10^{-6}	20 ± 0	1 ± 0
	10^{-5}	20 ± 10.4	0.5 ± 0.71
VW	0	20 ±10.4	0.5 ± 0.71
	10^{-6}	11.3 ± 21.61	0.5 ± 0.71
	5x 10^{-6}	20 ± 0	1 ± 0
	10^{-5}	20 ± 0	0.5 ± 0.71
Agar+Sucrose	0	27.6 ± 49.83	0.5 ± 0.71
	10^{-6}	11.3 ± 21.61	1 ± 1.4
	5x 10^{-6}	80 ± 10.4	1.5 ± 1.41
	10^{-5}	72.4 ± 49.83	2.5 ± 0.71

Incubated at 23-26° C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Number of seedlings per treatment = ten seedlings, divided into two replicates
Duration of observation for survival percentage = 4 weeks
Duration of observation for mean number of shoots = 12 weeks

(Plates 16b & 16c). All seedlings that survived on basal medium did not have any shoot proliferation. Seedlings that died included both seedlings that did and did not generate buds.

Although the mean numbers of shoot buds were not significantly different between all treatments (Appendix 30b), half-strength MS, half-strength GB5 and agar+sucrose, supplemented with 10^{-5} M BAP gave the highest mean numbers, respectively 3.0 ± 1.41 s.d., 2.5 ± 3.53 s.d. and 2.5 ± 0.71 s.d. (Table 8). The performances of seedlings cultured in media with lower concentrations of BAP were not different from those without BAP. The variability in the numbers of shoot buds produced within treatments was much less compared with the variability in survival percentages. This is perhaps not surprising as once the seedlings survived the establishment phase, their growth is influenced by the basal medium and the concentration of phytohormones.

6.3.2.1.2 Effects of BAP and basal media on the multiplication of shoot buds from apical shoots and nodal segments

Previous work by Rathore *et al.* (1991) and Latha & Seeni (1994) showed that nodal segments of *N. khasiana* were able to generate relatively large numbers of axillary shoots under suitable *in vitro* conditions. To determine whether this was possible with *N. macfarlanei*, an experiment, similar to experiment 6.3.2.1.1 was carried out, except that in place of cotyledonary seedlings, apical shoots and nodal segments were used. The apical shoots and nodal segments were obtained from seedlings with more than five leaves which had been cultured on semi-solid half-strength basal MS medium supplemented with $30gl^{-1}$ sucrose. The apical shoot of the plant was removed and the remaining stem was divided

Plates 16a-c : Multiplication of shoot buds from cotyledonary seedlings of *N. macfarlanei*

Plate 16a : Outgrowths (arrow) from the shoot apex

Plate 16b : Morphogenic development of outgrowths into shoot buds

Plate 16c : Development of shoot buds


16a
0.15cm


16b
0.5cm


16c
0.35cm

into nodal segments not more than 5 mm in length. Two replicates, each having five explants were used in all treatments. Observations were carried out over a period of three months.

Analysis of the results shows that the mean number of buds produced by apical shoots varied significantly between treatments ($P<0.01$, $n=10$) (Appendix 31). Apical shoots cultured in half-strength MS supplemented with $5x10^{-6}$ and 10^{-5} M BAP and half-strength GB5 supplemented with 10^{-6} M BAP gave the highest mean numbers of buds (Table 9). The former is in accordance with the results obtained from the use of cotyledonary seedlings as explants. In all treatments, there were no noticeable outgrowths on the shoot apices for the first two months. During this time, the shoot continued to produced new leaves. Only after two months, small outgrowths could be seen close to the apical region in media supplemented with BAP. The development of these outgrowths during subsequent subculture were similar to that of the outgrowths from the cotyledonary seedlings.

Nodal segments in all treatments (including the control *i.e.* full-strength MS) died and could not yield axillary shoots. All the segments turned brown and died before the period of observation was completed. The poor response could be due to the persistence effects of apical dominance on the segments and the presence of a rudimentary meristematic region in the axils of leaves. Casual field observations showed that *N. macfarlanei* does not often produce axillary shoots. If such factors do exist in the plant, it would probably require the use of a wider selection and combination of basal media and phytohormones than those investigated here to promote the development of the meristems. The results obtained are in contrast to those reported by Rathore *et al.* (1991) and Latha & Seeni (1994) for *N. khasiana.*

Table 9 : Bud development from apical shoots cultured on various basal media and BAP concentrations

Basal media	BAP (M)	Mean number of shoots ± s.d.
Full-strength MS	0	0
	10^{-6}	1 ± 1.4
	$5x10^{-6}$	0.5 ± 0.71
	10^{-5}	1 ± 1.4
Half-strength MS	0	0
	10^{-6}	1.5 ± 1.41
	$5x10^{-6}$	2.0 ± 0
	10^{-5}	2.5 ± 0.71
Full-strength GB5	0	0
	10^{-6}	0
	$5x10^{-6}$	1 ± 1.4
	10^{-5}	0.5 ± 1.07
Half-strength GB5	0	0
	10^{-6}	2.2 ± 0.35
	$5x10^{-6}$	0
	10^{-5}	0
NN	0	0
	10^{-6}	0
	$5x10^{-6}$	0
	10^{-5}	0
SH	0	0
	10^{-6}	0
	$5x10^{-6}$	0.5 ± 1.07
	10^{-5}	0
VW	0	0
	10^{-6}	0
	$5x10^{-6}$	0.5 ± 0.71
	10^{-5}	0
Agar+Sucrose	0	0
	10^{-6}	1.2 ± 0.35
	$5x10^{-6}$	0.5 ± 0.71
	10^{-5}	0

Incubated at 23-26° C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Number of seedlings per treatment = ten seedlings, divided into two replicates
Duration of observation for survival percentage = 4 weeks
Duration of observation for mean number of shoots = 12 weeks

6.3.2.2 To determine the optimal cytokinin and its concentration for the multiplication of shoot buds from cotyledonary seedlings

Results from experiment 6.3.2.1.1 shows that although the survival percentage of seedlings cultured on half-strength basal MS was significantly lower compared with those cultured on agar+sucrose, the mean number of shoot buds was not significantly different. On the assumption that all plants require some nutrient input for multiplication, half-strength basal MS was selected as the basal medium for experiments involving more prolonged growth. In addition, results from experiment 6.3.2.1.2 showed that cotyledonary seedlings were the superior explant for the multiplication of shoot buds; although the apical shoots were able to generate axillary shoots, they did so after a longer period of time compared with those from cotyledonary seedlings. Therefore, cotyledonary seedlings were chosen as the explant for subsequent experiments.

Apart from BAP, the other cytokinins tested were kinetin and 2-iP. The concentrations used for each cytokinin were 10^{-6}, 5 x 10^{-6} , 10^{-5}, 2.5 x 10^{-5} and 5 x 10^{-5} M. Four replicates of five cotyledonary seedlings each were used for each treatment and a completely randomized factorial design was employed. The seedlings were evaluated after 30 days for the percentage survival and the percentage producing outgrowths. Seedlings producing outgrowths were subsequently subcultured twice onto half-strength basal MS with $30gl^{-1}$ sucrose before the mean numbers of shoots were recorded. The duration of the observations was 3 months and the experiment was repeated once.

Analysis of results shows that the mean number of shoots derived from cotyledonary seedlings varied significantly between the media supplemented with different cytokinins ($P<0.001$, $n=20$)(Appendix 32). In both experiments, only BAP was able to induce multiple shoot formation; there were hardly any buds developing on seedlings cultured in medium

supplemented with 2-iP and kinetin (Table 10). Low BAP concentrations gave a much higher mean number compared with all concentrations of 2-iP and kinetin. Survival percentages were also significantly lower in 2-iP and kinetin (Table 10).

The rate of shoot multiplication was enhanced with the increase in BAP concentrations up to $5x10^{-5}$ M, the highest concentration tested. Once again, shoot outgrowths, which subsequently grew into buds, were restricted to the shoot apex region of seedlings. Outgrowths were observed within 30 days in media supplemented with BAP. No callus formation was observed on any parts of the seedling in any of the treatments. Excised buds subcultured onto half-strength basal MS did not root; they merely extended and produced several leaves. The ability of these shoots subsequently to root in media supplemented with auxins (see 6.3.4), however, indicated that high concentrations of BAP did not have long-term inhibitory effects on root initiation and formation.

Table 10 : Survival and bud development from seedlings cultured on half-strength MS supplemented with various concentrations of cytokinins

Cytokinin	Concentration (M)	Mean survival ± s.d. (%)	Mean number of shoots ± s.d.
BAP	10^{-6}	50 ± 11.6	1.3 ± 0.5
	5×10^{-6}	60 ± 28.3	2.9 ± 0.78
	10^{-5}	60 ± 16.3	3.6 ± 1.40
	2.5×10^{-5}	60 ± 43.2	4.2 ± 1.23
	5×10^{-5}	75 ± 19.2	6.7 ±1.25
2-iP	10^{-6}	5 ± 10	0.3 ± 0.5
	5×10^{-6}	0	0
	10^{-5}	6.6 ± 11.55	0.3 ± 0.58
	2.5×10^{-5}	5 ± 10	1 ± 2
	5×10^{-5}	5 ± 10	0.2 ± 0.5
Kinetin	10^{-6}	0	0
	5×10^{-6}	5 ± 10	1 ± 2
	10^{-5}	5 ± 10	1 ± 2
	2.5×10^{-5}	5 ± 10	0.3 ± 0.5
	5×10^{-5}	10 ± 11.6	1 ± 1.42

Incubated at 23-26°C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Number of seedlings per treatment = twenty, divided into four replicates
Duration of observation for survival percentage = 4 weeks
Duration of observation for mean number of shoots = 12 weeks

6.3.3 Somatic embryogenesis

To determine whether somatic embryogenesis could be of use as a multiplication tool for *N. macfarlanei*, an experiment was carried out using various auxins and explants. In the following experiments, the auxins used were 2,4-D and 2,4,5-T, in concentrations of 10^{-6}, $5x10^{-6}$ and 10^{-5} M and the basal media used were full-strength and half-strength MS, full-strength GB5, Nitsch & Nitsch, Schenk & Hildebrant and Vacin & Went; all of the media were supplemented with $30gl^{-1}$ sucrose and 8 gl^{-1} agar. The experiments involved a completely randomized factorial design. Cultures were incubated in light and in the dark (see 6.2.1.6).

The explants investigated included mature embryos, immature embryos, leaf segments and cotyledonary seedlings. In the experiment with immature and mature embryos, twenty embryos, without their seed coats and divided into four replicate batches were used for each treatment. Immature embryos which had developed approximately 8 weeks after pollination were used. These embryos were scored for survival and production of callus after 30 days in culture. This experiment was repeated once.

In the experiment with leaf segments, seedlings with more than five leaves were used as explants. The average size of a leaf segment was 5 x 5 mm, obtained from the midrib region of the leaf. For each treatment, fifty segments were used, divided into five replicate batches. Preliminary experiments had showed that the position of the segment taken from the leaf did not affect its survival percentage.

In the experiment with cotyledonary seedlings, only half-strength basal MS was used and the concentrations of 2,4-D and 2,4,5-T were increased to 2.5 x 10^{-5} and 5 x 10^{-5} M

respectively. The seedlings were evaluated for callus and somatic embryos after 30 days in culture. This experiment was repeated once.

Analysis of results shows that all explants, with the exception of cotyledonary seedlings, did not survive nor produce any callus in treatments supplemented with 2,4-D and 2,4,5-T (Appendix 33). Explants cultured on basal media, with the exception of leaf segments, survived but did not yield any callus. Only cotyledonary seedlings showed some survival in 10^{-6} M 2,4-D and 10^{-6} M 2,4,5-T; the survival were 20% and 40% respectively.

All explants that were incubated in the dark, irrespective of auxin incorporation, died within 2 weeks after culture. Those that were maintained under light but died, did so within 4 weeks after culture. At death, there were no visible signs of callus formation on any part of the explant. Explants that survived, did not produce any callus when the medium had no auxin. Cotyledonary seedlings that survived on media supplemented with auxin did not produce any callus either, indicating that 2,4-D and 2,4,5-T were not effective. The response to 2,4-D and 2,4,5-T obviously differed with the explant used. 2,4-D and 2,4,5-T were detrimental to immature and mature embryos and leaf segments but at low concentration, they were not harmful to cotyledonary seedlings.

6.3.4 Rooting of shoot buds

Axillary shoots derived from the shoot apex region of cotyledonary seedlings and apical shoots (see 6.3.2.1.1, 6.3.2.1.2 and 6.3.2.2) were separated and transferred onto half-strength basal MS medium with 30 gl^{-1} sucrose & 8 gl^{-1} agar. They were maintained in this medium for one month. Subsequently, NAA, IBA and IAA, each at concentrations of 10^{-6}, $5x10^{-6}$ and 10^{-5} M were used to determine the most suitable auxin and its concentration for rooting. The basal medium used was similar to the above. Twenty replicates, divided into

five petri dishes were used for each treatment and a completely randomized design was employed. After incubation for three months in light, the plantlets were scored for callus and root formation.

Analysis of results shows that callus and roots were readily produced in media supplemented with auxins (Tables 11 & 12). Although the survival percentage of shoots were lowest in all NAA treatments, 100% of the shoots that survived rooted while in other auxin treatments, there were proportions that did not root. 10^{-5} M NAA gave the highest frequency of plants bearing more than 20 roots and $5x10^{-6}$ M IAA gave the highest frequency of plants bearing roots more than 3.0 mm long (Table 11).

These roots develop mostly from callus and occasionally they developed from the cut end of the excised shoot, without any apparent sign of callus formation (Plates 17a & 17b). The roots were morphologically similar to tap-roots and they were covered with numerous fine hairs. None of the rooted seedlings developed lateral roots. In media supplemented with $5x10^{-6}$ M IBA, roots grew to 1 cm long during the experimental period.

The size of callus varied between treatments (Table 12). All NAA treatments induced large basal callus formation and the largest proliferation was observed in 10^{-5} M NAA (Plate 17b) while very little callus was observed on $5x10^{-6}$ and 10^{-5} M IAA. All calluses were compact and black and they only became visible after approximately 8 weeks in culture.

The production of large basal callus by excised shoots on media supplemented with NAA implied that this auxin might be used for callus induction and shoot formation via organogenesis, perhaps when used in combination with suitable concentrations of a cytokinin.

Table 11 : Rooting frequency of axillary shoots cultured on half-strength MS supplemented with various concentrations of auxins

Auxin	concen-tration (M)	No. of surviving shoots	Root								
			length (mm)					number			
			0	<1	1.1- 2.0	2.1 - 3.0	>3.0	0	<10	11 - 20	>20
IAA	10^{-6}	11	3	1	3	2	2	3	3	3	2
	$5x10^{-6}$	8	2	2	1	1	2	2	3	3	0
	10^{-5}	6	3	0	0	3	0	3	2	1	0
IBA	10^{-6}	10	7	2	0	0	1	7	2	0	1
	$5x10^{-6}$	15	5	0	6	1	3	4	5	5	1
	10^{-5}	9	5	0	2	0	2	5	1	1	2
NAA	10^{-6}	7	0	3	2	2	0	0	2	3	2
	$5x10^{-6}$	5	0	0	3	2	0	0	0	2	3
	10^{-5}	8	0	1	4	1	2	0	1	1	6

Total number of shoots per treatment = twenty, divided into four petri dishes
Incubated at 23-26°C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Duration of observation for root formation = 12 weeks

Table 12 : Callusing frequency of axillary shoots cultured on half-strength MS supplemented with various concentrations of auxins

Auxin	concen-tration (M)	No. of surviving shoots	Size of callus (cm)						
			nil	0.1x0.1	0.2x0.2	0.3x0.3	0.4x0.4	0.5x0.5	>0.5x0.5
IAA	10^{-6}	11	0	4	2	1	2	1	1
	$5x10^{-6}$	8	1	6	1	0	0	0	0
	10^{-5}	6	0	5	1	0	0	0	0
IBA	10^{-6}	10	3	2	1	0	4	0	0
	$5x10^{-6}$	15	1	6	2	2	1	3	0
	10^{-5}	9	2	3	1	0	1	1	1
NAA	10^{-6}	7	0	0	3	0	1	2	1
	$5x10^{-6}$	5	0	0	0	0	1	0	4
	10^{-5}	8	0	0	0	0	0	0	8

Incubated at 23-26°C, 12 hours photoperiod , PAR 12-15 $\mu Em^{-2}s^{-1}$, RH 67-75%
Total number of shoots per treatment = twenty, divided into four petri dishes
Duration of observation for callus formation = 12 weeks

Plates 17a-b : Rooting of *N. macfarlanei* shoot buds

Plate 17a : Rooting in half-strength basal MS medium supplemented with 10^{-6} M IAA

Plate 17b : Rooting in half-strength basal MS medium supplemented with 10^{-5} M NAA


17a
1.0CM
½MS 10⁻⁶ IAA 17/2/95


17b
1.0CM
½MS 10⁻⁵ NAA 19/2/95

6.3.5 Weaning of plantlets

The rooted plantlets were removed from the culture vessels and rinsed under running water to wash off the agar. A prepared mixture of M2 compost without added fertilizer was used to fill plastic trays measuring 5x5x6 cm. The plantlets were transferred to a 24 ± 2°C glasshouse and watered daily. After one month, each plantlet was scored for survival and the number of newly-developed leaves. As all the surviving plantlets from the rooting experiment (see 6.3.4) were used, the actual number of replicates varied according to the previous auxin treatments. A completely randomized design was used.

Observations shows that 100% of the plantlets survived when transferred to the compost mix. By the end of the first month, all of them had developed a healthy young shoot with at least one new leaf while the old ones withered and died.

6.4 DISCUSSION

N. macfarlanei seeds require a simple substrate for germination. Unlike many other plant species, the use of a more complex combination of macro and micro salts prohibits germination. This germination performance is in accordance with the fundamental principle that a seed is an independent and self-sustaining propagule. In the case of *N. macfarlanei*, the embryonic axis is entirely dependent on the cotyledons for nutrients since there is no persistent endosperm. For practical purposes, the use of a simple medium for germination is advantageous. Sucrose and agar are basic components of most media and are easily obtained.

Despite the ability to germinate in a simple medium, the time taken for axenic seeds to germinate was much greater compared with that of fresh seeds (Table 7). Many of the seed batches used had been previously stored in 4°C; results from seed viability studies show that there was a major decline in the viability of stored seeds (see Chapter 5 for more details). This could have influenced the extension in germination time, apart from other factors such as intrinsic properties of the seed batches used and the effects of surface sterilization and culture medium. A similar slow response was also noted in *N. madagascariensis* and *N. carunculata* by Redwood & Bowling (1990), although it is not known whether seeds of these species had been stored prior to use.

Half-strength MS, supplemented with at least 10^{-5} M BAP (higher concentrations of up to 5×10^{-5} M are beneficial) was the optimal medium out of those tested for shoot multiplication (Tables 8 & 9) while the same medium supplemented with NAA was optimal for the rooting of shoot buds (Table 10). It is worth noting that these media are fairly simple and the use of a more complex medium such as advocated by Rathore *et al.* (1991) for *N. khasiana* would be rather impractical because laboratories used for the purpose of

plant rescue, restoration and re-introduction are often not as well-equipped as more advanced research laboratories because of the lack of funds. In areas where there is a high degree of endemism, the resource managers would often have to recover and multiply many rare/endangered species occurring in their area of jurisdiction. Therefore, for practical purposes, they need a relatively simple protocol, using either the *in vitro* or the *in vivo* method, that can be applied, with some degree of success, to the genus that contains rare or endangered species. With such an aim in mind, one would be primarily inclined towards producing sufficient amounts for restoration, re-introduction and perhaps distribution to botanic gardens and not towards producing large bulks, at least in the first instance. The latter aim might become important as part of a more general conservation strategy.

The multiplication protocol developed for *N. macfarlanei* may also serve as guide for the *in vitro* propagation of a wider range of *Nepenthes* spp., particularly those that are highly endemic and rare (*e.g. N. rajah* and *N. villosa* in Mt. Kinabalu, Sabah, Borneo). However, as aptly remarked by Maunder (1991), plants rescued and used as stocks for re-introduction are likely to have a narrow genetic base. Although it is not unusual for isolated wild populations to originate from a few founder individuals (Schwaegerle & Schaal, 1979; Frankel & Soule, 1981; Barrett & Kohn, 1991) and appear to possess, in the short term, no perceptible lack of vigour, the long term effects on the regenerating population must be considered. The use of seedling explants is recommended as they are heterogeneous. However, in many species, the supply of seeds is often restricted. As such, although the use of *N. macfarlanei* cotyledonary seedlings as explants is advantageous because it reduces the time required to initiate shoot multiplication and all seedlings are of unique genotypes, the study into the propagation of other explants as attempted by this work should be pursued.

6.5 CONCLUSION

N. macfarlanei may be easily propagated through the *in vitro* technique. However, at the present time, the multiplication protocol developed is perhaps more useful as a guide for the rescue and recovery of other endangered/rare *Nepenthes* sp. since *N. macfarlanei* is not at present endangered or threatened in its natural habitats. When used for other species of interest, it is likely that this protocol will need some modification, particularly in the concentrations of cytokinins and auxins.

CHAPTER 7

FINAL CONCLUSION

FINAL CONCLUSION

7.1 CONSERVATION STATUS

This study shows that *Nepenthes macfarlanei* Hemsl. has a narrow geographical range and habitat specificity. It is distributed in massifs located in the central and western parts of Peninsular Malaysia but restricted to certain peaks within the massifs. It is confined to the lower and upper montane forest formations and is not found in other forest formations (following the forest classification for Malaysia).

Although disjunct in distribution because of populations being confined to certain mountain peaks, *N. macfarlanei* is locally abundant within its habitats. This seems to indicate that it is able to maintain certain levels of viability in face of the stochastic forces acting on its populations. Results from the population and reproductive studies at two separate experimental sites in the Main Range seem to be in accordance with the above suggestion; these studies indicate that this species possesses some basic requirements needed to maintain sufficient levels of survival and viability in its population and these include high regeneration and establishment capacities of juveniles, the ability to tolerate environmental extremities in its habitats, the interaction with a diverse group of insect pollinators and the production of a large number of viable seeds. The ability of this species to sustain its population is most clearly seen in several localities where forest fragmentation had occurred such as at the summits in Cameron Highlands. The development of the land there for agriculture must have had affected its population sizes but, at present, those remaining are still regenerating.

In view of this, the species *per se* may not be considered vulnerable at the present time, although some isolated populations may come under threat. However, this status is largely

determined by prevailing population densities and naturally-occurring stochastic forces. The latter could result from genetic, demographic and environmental factors and from human intervention. The factors which are particularly relevant to *N. macfarlanei* include the fragmentation of the habitat and resulting changes in the physical environment. Although the present study indicates that the populations in fragmented forests are apparently thriving, it is, however, not known whether the current sizes of the populations are below or above the critical point.

Fragmentation of natural habitats into smaller sizes and the loss in habitat continuity is fast gaining pace in 'hotspots' (countries with mega-diversity). In Peninsular Malaysia, although reliable values in terms of hectarage are not currently available (as of late 1995), fragmentation of the upper montane forests is confined to the southern limit and mid-portion of the Main Range, leaving forests and species diversity in other parts of that massif and in other massifs very much intact. Although it can be assumed that some portions of the genepool in populations occurring in those areas will have disappeared, it is not known to what degree has it been lost and to what extent the present genepool is representative of the original.

7.2 RECOMMENDATIONS FOR CONSERVATION

7.2.1 *In situ* measures

In the attempt to develop strategies for the *in situ* conservation of *N. macfarlanei*, three crucial attributes of the species must be given particular attention. The first is its mutualistic interactions with small generalist insects for pollination; this may be an advantage if the species is not highly dependent on the presence of very specific insects, nevertheless changes in the extrinsic factors that lead to drastic alterations in the viability and composition of the insect populations could have direct consequences on the reproductive

ability and regeneration of its populations. Although this study was unable to compare the frequency of effective pollinator visitation with the RE (reproductive efficiency) of the sample population, the low RE observed suggests that the frequency of visitation does influence the reproductive capacity of the mature individuals. The second attribute is its allogamous breeding system. The studies clearly demonstrated that it is an obligate outbreeder, and therefore it can be assumed that reduced population densities brought about by the loss of habitat could eventually lead to lower rates of reproduction. The third attribute is its seed dispersal mechanism. Although *N. macfarlanei* has been shown to possess a number of survival attributes, the lack of individuals between closely occurring populations suggests that colonization into new areas with similar habitats does not occur readily, and therefore the normal turnover of plants in a declining population would not be easily supplemented by immigration from other populations that are still extant.

Based on the above findings and the recognition that in Malaysia, conservation of biological diversity competes with the nation's socio-economic needs, it is suggested that future fragmentation of the montane forests for the purpose of development such as for tourist resorts and the construction of roads be confined to localities already experiencing fragmentation. As the occurrence of *N. macfarlanei* is predominantly at the Main Range, it is imperative that other parts of the Range, as well as other ranges, be left undisturbed to ensure that minimum population sizes and genetic diversity may be maintained. The conservation of the montane habitats for *N. macfarlanei* has many advantages, of which the most important ones are the conservation of a highly endemic montane flora, of which this species is typical, and the maintenance of watershed areas.

This recommendation is not only suggested for *N. macfarlanei* but also for the rest of the endemic *Nepenthes* spp. In the case of hyper-endemics, it is of paramount importance to

maintain all the areas where they occur for, although their small populations will occasionally suffer from random extinction, further reductions in population sizes will only accelerate the rate of extinction. In all aspects, the extremely narrow geographic range and the restriction to a limited number of localities place the species at a greater risk of extinction. Fortunately, in Malaysia, where hyper-endemics are concerned, there have been moves to give them the necessary legal attention and conservation measures and the totally protected national park status has been given to most areas where they occur.

7.2.2 *Ex situ* measures

The widespread and locally abundant distribution of *N. macfarlanei* implies that, provided its habitats do not continue to fragment further, *in situ* conservation measures can probably safe-guard its populations. The application of *ex situ* techniques in the germplasm conservation of *N. macfarlanei* is not considered critical at the present time. The micropropagation protocol developed in this study may be applied to *N. macfarlanei* and probably, with modifications, to other *Nepenthes* sp., particularly in cases where the rescue of threatened populations and their recovery and restoration are important. However, the use of seed long-term storage for germplasm conservation cannot be recommended for *N. macfarlanei* at the present time due to the relatively poor viability of the stored seeds. Further research is required (see below) before this technique could be recommended as a potential strategy.

7.2.3 Administrative framework

At present, much of the Highland region in Peninsular Malaysia is under the forest reserve system and therefore under the jurisdiction of the state governments. Unfortunately, past experiences have demonstrated a lack of appreciation of the importance of highland

habitats both in terms of their biological diversity and the maintenance of basic ecological functions, resulting in a minimal regard for the need to protect such forests. This attitude primarily arises from the lack of information and the poor information transfer between research and planners. Recognizing these limitations, the draft National Policy on Biological Diversity suggests, as part of the action plan of programmes, more extensive research and an update of information on the country's wealth of biological diversity and stronger linkages with the state bureaucracies so that all parties may be involved in the planning and implementation of conservation measures related to the sustainability of biological diversity.

7.2.4 Future research

In view of the recommendation listed in the draft National Policy relating to the need for more research, the following are recommendations for further studies with application to *N. macfarlanei* in particular and *Nepenthes* spp. in general :

(i) Investigations into the minimum population sizes required by a species to maintain long-term survival. Each species is likely to differ in this requirement, therefore, priority should be aimed at the hyper-endemic species. The studies on the rates of gene flow by pollen and seed, the derivation of extinction probabilities, the effect of small population sizes on the genetic diversity will be able to contribute to the determination and demarcation of areas for conservation.

(ii) Investigations into the long-term storage and viability of seeds. The present study was neither able to provide conclusions nor introduce protocols that would be useful for the long-term germplasm storage of *N. macfarlanei*. Detailed work is, therefore, recommended on the regulation of moisture content of seeds during storage and the use of low temperatures (including cryopreservation) and ultra-dry conditions.

(iii) Investigations into the somatic embryogenesis and *in vitro* multiplication of shoot buds using nodal segments as explants. The present study was unable to provide protocols for the regeneration of shoot buds from nodal segments and somatic embryos. Detailed work is, therefore, recommended on the use of other basal media and phytohormones.

(iv) Investigations into the genetic diversity of *Nepenthes* sp., particularly of hyper-endemics. The genetic diversity of a population and the comparison of diversity between populations will provide the data required to determine whether present population sizes have reached critical levels. Detailed work is recommended on the genetic diversity of populations using molecular techniques.

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APPENDIX

Appendix 1 : Sources of trade for the carnivorous group of plants between 1983-1989 (Number in parentheses indicates the percentage of plants derived from artificial propagation) (World Conservation Monitoring Centre, 1991)

Primary exporter	Term	Quantity
Australia	live	513 (99.81%)
Brunei Darussalam	live	276 (40.58%)
Canada	live	1 (100%)
France	live	44 (100%)
Germany	live	1 (100%)
Malaysia	live	28 (39.39%)
Netherlands	live	3355 (100%)
Philippines	live	172 (97.67%)
Thailand	live	4 (50%)
United States	live	22626 (50.14%)
-do-	roots	7143
-do-	pieces	32006

Appendix 2 : The known distribution of *N. macfarlanei* in Peninsular Malaysia

Collector	Number	Collection date	State and locality	Habitat
n.a.	18562	4/1911	Selangor, Mt. Semangkuk	n.a.
n.a.	KEP66582	24/8/1949	Pahang, Mt. Jasar	summit, secondary elfin forest, 1525m
Wyatt-Smith J.	KEP94563	15/5/1960	Selangor, Mt. Tunggal	summit, primary forest, 1660m
n.a.	s.n.	10/1877	Perak, Mt. Bubu	1525m
Abang Mohtar A.P.	88	4/3/1979	Pahang, Genting Highlands	1830m
Asmah T.	103	16/1/1979	Pahang, Mt. Brinchang	on peat, 2030m
Burkill H.M.	HMB2382	18/8/1960	Pahang, Pine Tree Hill	exposed rocky scrub, 1450m
Burkill H.M.	HMB787	1/9/1956	Pahang, Mt. Batu	exposed summit ridge, 2030m
Flenley J.R.	29	16/2/1969	Selangor, Mt. Ulu Kali	summit plateau on mossy peat, 1770m
Haniff & Nur	8306	22/6/1922	Pahang, Mt. Tahan	1220-1830m
Hashim M.	51	17/1/1979	Pahang, Mt. Brinchang	n.a.
Hashim M.	52	16/1/1978	Pahang, Mt. Brinchang	2030m
Holttum R.E.	SFN31274	16/5/1936	Pahang, Mt. Batu	summit clearing, 2030m
Holttum R.E.	SFN31275	15/5/1936	Pahang, Mt. Batu	2000m
Hullett R.W.	874	8/1888	Malacca, Mt. Ophir	summit
Wyatt-Smith J.	KEP79152	29/3/1958	Pahang, Mt. Mengkuang	open ridge, 1495m
Jaamat	KEP27026	10/8/1931	Pahang, Batu Gangan	summit hill
Kasim M.	055	8/1/1979	Pahang, Mt. Brinchang	1830m
Kassim	s.n.	7/7/1974	Pahang, Genting Highlands	2440m
King	7395	3/1883	Perak, Mt. Bubu	summit, open jungle
King	7421	3/1885	n.a.	1465-1620m
Landon J.H.	KEP45159	31/1/1938	Perak, G. Bubu	summit, 1655m
Littke W.	WL349	30/3/1974	Pahang, Fraser's Hill	roadside, 1340m
Melville R. & Landon F.H.	4821	30/8/1953	Pahang, Pine Tree Hill	summit, 1460m
Mohd Kasim R.	MK+ZA11011	11/1/1974	Pahang, Genting Highlands	n.a.
Allen M.	4933	5/4/1963	Pahang, Mt. Brinchang	2030m
Ng F.S.P.	FRI020961	2/3/1973	Pahang, trail to Mt. Tahan	ridge, 1525m
Noh M.	02116	21/7/1974	Selangor, Mt. Bunga Buah	secondary outgrowth, 1190m
Nur Md.	12221	6/3/1924	Kelantan, Mt. Stong	ridge, 790m
Rahim A.	052	16/1/1979	Pahang, Mt. Brinchang	on peat, 2010m
Ridley H.N.	s.n.	8/1909	Selangor, Ulu Semangkuk	n.a.
Robinson H.C.	s.n.	6/1913	Mt. Kerbau (state unknown)	2010m
Samat A.	UKMB 00263	20/12/1970	Pahang, Cameron Highlands	n.a.
Shah M.	MS2961	11/6/1973	Selangor, Mt. Ulu Kali	1770m
Sow	47175	19/6/1938	Perak, Mt. Raya	summit hill
Strugnell E.J.	KEP45891	1/2/1938	Perak, Mt. Korbu	2180m
Symington C.F.	KEP20820	24/5/1931	Pahang, Rhododendron Hill	n.a.
Symington C.F.	KEP20927	13/10/1929	Pahang, Rhododendron Hill	1370m
Symington C.F.	KEP30848	8/4/1933	Perak, Mt. Bubu	summit
Symington C.F.	KEP32123	22/7/1933	Perak, Mt. Korbu	summit ridge, mossy forest
Symington C.F.	KEP32137	22/7/1933	Perak, Mt. Korbu	ridge, 1680m
Symington C.F.	KEP32220	22/7/1933	Perak, Mt. Korbu	mossy forest, 1830m
Symington C.F.	KEP32221	22/7/1933	Perak, Mt. Korbu	open ridge, 1830m
Symington C.F.	KEP36226	12/4/1934	Pahang, Mt. Terbakar	summit
Symington C.F.	KEP36546	14/4/1934	Pahang, Mt. Irau	summit
Symington C.F.	KEP11541	20/5/1931	Pahang, Batu Gangan	1905m
Wong Y.K. & Wyatt-Smith J.	W60	2/1961	Pahang, ascent to Mt. Tahan	n.a.
Wray	1643	n.a.	Pahang, Mt. Batu Putih	nil
Wray	339	23/2/1909	Mt. Bulu Patik (state unknown)	summit, 2045m
Wray	3846	3/1890	Perak, Mt. Bubu	1525m
Wray	3849	3/1890	Perak, Mt. Bubu	1525m

Appendix 3 : List of plant species found in the sample plots at Cameron Highlands

Species	Family
Rhododendron malayanum	Ericaceae
R. javanicum	Ericaceae
R. wrayi	Ericaceae
R. perakense	Ericaceae
Vaccinum scortechinii	Ericaceae
V. loranthifolium	Ericaceae
V. viscifolium	Ericaceae
Elaeocarpus nanus	Elaeocarpaceae
E. stipularis	Elaeocarpaceae
E. nitidus v. nitidus	Elaeocarpaceae
E. latifolia	Elaeocarpaceae
Cinnamomun subavenicum	Lauraceae
C. porrectum	Lauraceae
Eugenia sp.	Myrtaceae
Leptospermum flavescens	Myrtaceae
Gordonia scortechinii	Theaceae
Garcinia cantleyana	Guttiferae
Mesua wrayi	Guttiferae
Euodia latifolia	Rutaceae
Ficus deltoides	Moraceae
Schefflera ridlyei	Araliaceae
Pinanga polymorpha	Palmae
Bambusa sp.	Graminae
Lithocarpus ewyckii	Fagaceae
Ardisia retinervia	Myrsinaceae
Garcinia hombroniana	Guttiferae
Chonianthus caudifolius	Oleaceae
Lasianthus scalariformis	Rubiaceae
Elaeocarpus glabrescens	Elaeocarpaceae
Oxyspora acutangula	Melastomataceae
Nepenthes sanguinea	Nepenthaceae
Cratoxylum maingayi	Hypericaceae
Elaeocarpus symingtonii	Elaeocarpaceae

Appendix 4 : List of plant species found in the sample plots at Mt. Purun

Species	Family
Melastoma muticum	Melastomataceae
Rhododendron robinsonii	Ericaceae
Rhododendron malayanum	Ericaceae
Rhododendron scortechinii	Ericaceae
Vaccinium viscifolium	Ericaceae
Gaultheria leucocarpa	Ericaceae
Symingtonia populnea	Hamamelidaceae
Weinmania blumei	Cunoniaceae
Dacrydium comosum	Podocarpaceae
Eurya nitida	Theaceae
Ficus deltoides	Moraceae
Rhodamnia cinerea	Myrtaceae
Elaeocarpus nanus	Elaeocarpaceae
Calophyllum rotundifolium	Guttiferae
Garcinia cantleyana	Guttiferae
Medinella scortechinii	Melastomataceae
Eugenia oreophila	Myrtaceae
Acronychia magnifica	Rutaceae
Sonerila hirsuta	Melastomataceae
Schima walichii	Theaceae
Gordonia imbricata	Theaceae
Leptospermum flavescens	Myrtaceae
Prunus arborea	Rosaceae
Pandanus klosii	Pandanaceae
Dipteris conjugata	Pteridophyta
Matonia pectinata	Pteridophyta
Pinanga polymorpha	Palmae
Bambusa sp.	Graminea
Argostemma yappi	Rubiaceae
Nepenthes sanguinea	Nepenthaceae
Nepenthes gracillima	Nepenthaceae
Dianella javanica	Liliaceae
Curculigo latifolia	Hypoxidaceae
Didymocarpus oblanceolata	Gesneriaceae

Appendix 5 : The chi-square test on the density of *N. macfarlanei* in the sample plots at CH

No. of plants in plot	Observed frequency	No. of plants in freq. class	Expected frequency	Chi-square
0-10	56	112	5.72	441.97
11-12	4	46	9.53	3.21
13	2	26	7.1	3.66
14	1	14	8.45	6.57
15	1	15	9.39	7.5
16	3	48	9.79	4.71
17	2	34	9.6	6.02
18	1	18	8.89	7
19	1	19	7.8	5.93
20	1	20	6.5	4.65
21	1	21	5.16	3.35
22 23	1	22	6.74	4.89
>23	26	1272	5.32	80.39
Sum	100	1667	99.99	579.85
Mean		16.67		

$P<0.001$; $df = 11$

Appendix 6 : The chi-square test on the density of *N.macfarlanei* in the sample plots at Mt. Purun

No. of plants in plot	Observed frequency	Expected frequency	Chi-square
0-1	42	10.96	87.91
2	0	13.36	13.36
3	5	14.25	6
4	3	11.4	6.19
5	0	7.3	7.3
>5	14	6.73	7.85
Sum	64	64	128.61

$P<0.001$; $df = 4$

Appendix 7 : The variance:mean ratio of the density of *N. macfarlanei* in the sample plots at CH

Plot	No. of plants	Plot (cont.)	No. of plants	Plot (cont.)	No. of plants
1	1	34	5	67	93
2	0	35	3	68	97
3	17	36	0	69	109
4	5	37	0	70	6
5	14	38	1	71	18
6	44	39	0	72	4
7	3	40	0	73	11
8	8	41	0	74	0
9	34	42	0	75	5
10	32	43	0	76	9
11	29	44	0	77	13
12	13	45	0	78	48
13	7	46	0	79	59
14	0	47	0	80	54
15	16	48	0	81	54
16	88	49	0	82	28
17	6	50	0	83	45
18	0	51	0	84	47
19	2	52	0	85	24
20	4	53	0	86	29
21	12	54	0	87	36
22	16	55	0	88	21
23	11	56	0	89	49
24	6	57	0	90	54
25	0	58	1	91	32
26	1	59	2	92	3
27	0	60	4	93	12
28	0	61	22	94	48
29	0	62	16	95	27
30	0	63	5	96	30
31	1	64	9	97	44
32	6	65	15	98	19
33	20	66	17	99	38
				100	5

Sum 1667
Mean 16.67
Variance 536.6677778
Variance:mean ratio = 32.19

Appendix 8 : The variance:mean ratio of the density of *N. macfarlanei* in the sample plots at Mt. Purun

Plot(10x10m)	Terrain	No. of plants	Plot (cont.)	Terrain	No. of plants
1	steep-ledge	0	33	steep	0
2	steep-ledge	7	34	steep	0
3	steep-ledge	3	35	steep	0
4	steep-ledge	4	36	steep	0
5	steep-ledge	0	37	plateau	0
6	steep-ledge	3	38	plateau	0
7	steep-ledge	14	39	plateau	0
8	steep-ledge	22	40	plateau	1
9	steep	14	41	gentle	0
10	steep-plateau	0	42	gentle	0
11	steep-plateau	4	43	gentle	0
12	steep-plateau	11	44	gentle	0
13	steep	4	45	steep	0
14	steep	0	46	steep	0
15	steep	11	47	steep	0
16	steep	6	48	steep	0
17	steep	12	49	steep	0
18	steep	1	50	steep	0
19	steep	0	51	steep	0
20	plateau	3	52	steep	0
21	plateau	0	53	gentle	0
22	plateau	24	54	gentle	0
23	plateau	3	55	gentle	0
24	gentle	3	56	gentle	0
25	gentle	1	57	steep	0
26	plateau	9	58	steep	0
27	plateau	20	59	steep	0
28	plateau	11	60	steep	0
29	steep	8	61	steep	0
30	steep	0	62	steep	0
31	steep	1	63	steep	0
32	steep	6	64	steep	0

Mean = 3.21875
Variance = 32.6815
Variance:mean ratio = 10.181

Gentle = slope of < 45 degrees
Steep = slope of > 45 degrees

Appendix 9 : The increment in the variance:mean ratio with plot size at Mt. Purun (see also Appendix 8)

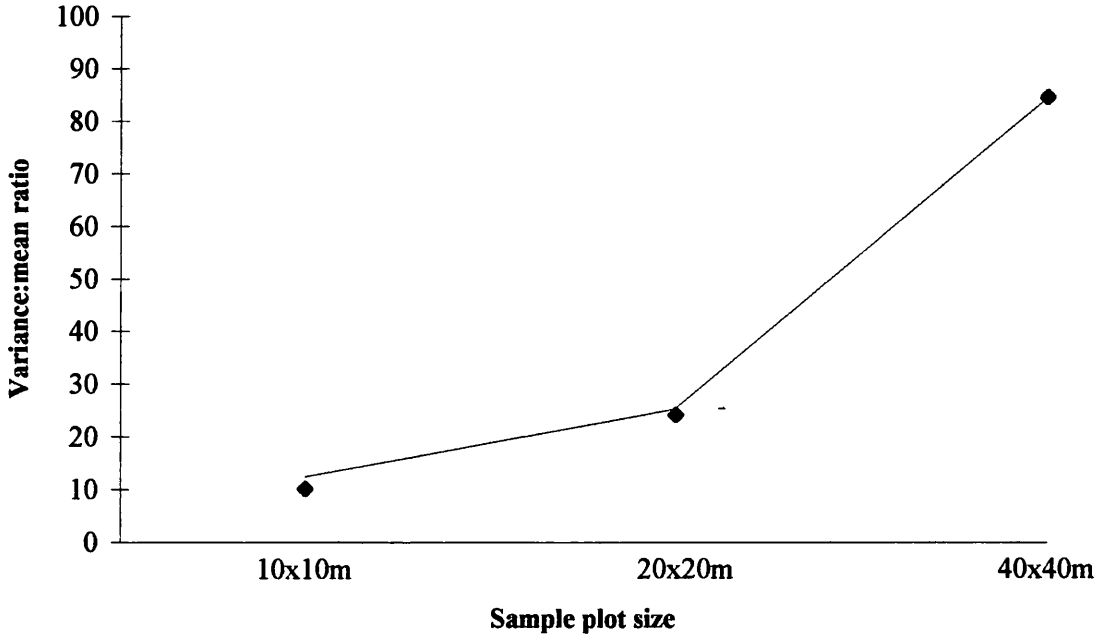
Plot (20x20 m)	No. of plants
64,63,49,50	0
62,61,51,52	0
60,59,53,54	0
58,57,55,56	0
48,47,33,34	0
46,45,35,36	0
44,43,37,38	0
42,41,39,40	1
32,31,17,18	20
30,29,19,20	11
28,27,21,22	55
26,25,23,24	16
16,15,01,02	24
14,13,03,04	11
12,11,05,06	18
10,09,07,08	50

Mean = 12.875
Variance = 311.45
Variance:mean ratio = 24.19

Plot (40x40 m)	No. of plants
64,63,62,61,49,50,51,52,48,47,46,45,33,34,35,3	0
60,59,58,57,53,54,55,56,44,43,42,41,37,38,39,4	1
32,31,30,29,17,18,19,20,16,15,14,13,01,02,03,0	66
28,27,26,25,21,22,23,24,12,11,10,09,05,06,07,0	139

Mean = 51.5
Variance = 4356.3333
Variance:mean ratio = 84.59

The increment in the variance:mean ratio with plot size at Mt.Purun



Appendix 10 : The density of *N. macfarlanei* plants in each sample plot at CH

Plot	Terrain	Zone code	Total no.	HC1	HC2	HC3	HC4	HC5	HC6
1	ridge	1	1	1	0	0	0	0	0
2	ridge	1	0	0	0	0	0	0	0
3	ridge	1	17	6	3	2	0	2	4
4	ridge	1	5	5	0	0	0	0	0
5	ridge	1	14	7	2	4	1	0	0
6	ridge	1	44	10	13	9	2	6	4
7	ridge	1	3	1	0	0	0	0	2
8	ridge	1	8	5	0	1	0	0	2
9	ridge	1	34	17	4	6	2	2	3
10	ridge	1	32	21	6	2	0	1	2
11	ridge	1	29	10	4	7	3	2	3
12	ridge	1	13	6	3	4	0	0	0
13	ridge	1	7	2	2	1	0	0	2
14	ridge	1	0	0	0	0	0	0	0
15	ridge	1	16	3	2	2	0	0	9
16	ridge	1	88	33	25	21	2	4	3
17	ridge	1	6	1	1	0	0	0	4
18	ridge	1	0	0	0	0	0	0	0
19	ridge	1	2	0	0	1	0	0	1
20	ridge	1	4	2	1	0	0	0	1
21	ridge	1	12	5	3	2	0	0	2
22	ridge	1	16	6	5	4	1	0	0
23	ridge	1	11	2	5	2	1	1	0
24	ridge	1	6	5	0	1	0	0	0
25	ridge	1	0	0	0	0	0	0	0
26	ridge	1	1	0	0	0	0	0	1
27	ridge	1	0	0	0	0	0	0	0
28	ridge	1	0	0	0	0	0	0	0
29	ridge	1	0	0	0	0	0	0	0
30	ridge	1	0	0	0	0	0	0	0
31	ridge	1	1	1	0	0	0	0	0
32	ridge	1	6	0	0	0	0	0	6
33	ridge	1	20	6	6	4	0	0	4
34	ridge	1	5	0	2	1	0	0	2
35	valley	2	3	2	0	1	0	0	0
36	valley	2	0	0	0	0	0	0	0
37	valley	2	0	0	0	0	0	0	0
38	valley	2	1	0	1	0	0	0	0
39	valley	2	0	0	0	0	0	0	0
40	valley	2	0	0	0	0	0	0	0

continue overleaf

41	valley	2	0	0	0	0	0	0	0
42	valley	2	0	0	0	0	0	0	0
43	valley	2	0	0	0	0	0	0	0
44	valley	2	0	0	0	0	0	0	0
45	valley	2	0	0	0	0	0	0	0
46	valley	2	0	0	0	0	0	0	0
47	valley	2	0	0	0	0	0	0	0
48	valley	2	0	0	0	0	0	0	0
49	valley	2	0	0	0	0	0	0	0
50	valley	2	0	0	0	0	0	0	0
51	valley bottom	2	0	0	0	0	0	0	0
52	valley bottom	2	0	0	0	0	0	0	0
53	valley bottom	2	0	0	0	0	0	0	0
54	valley	2	0	0	0	0	0	0	0
55	valley	2	0	0	0	0	0	0	0
56	valley	2	0	0	0	0	0	0	0
57	valley	2	0	0	0	0	0	0	0
58	valley	2	1	0	1	0	0	0	0
59	valley	2	2	0	0	0	0	0	2
60	valley	2	4	0	1	3	0	0	0
61	valley	2	22	6	2	3	1	0	10
62	valley	2	16	8	1	4	0	0	3
63	valley	2	5	1	0	2	0	0	2
64	valley	2	9	2	1	4	0	1	1
65	valley	2	15	1	8	3	1	0	2
66	valley	2	17	8	4	2	0	0	3
** 67	plateau	3	93	52	26	10	5	0	0
** 68	plateau	3	97	71	24	0	2	0	0
** 69	plateau	3	109	99	6	4	0	0	0
70	ridge	1	6	0	3	1	0	1	1
71	ridge	1	18	6	5	5	1	1	0
72	ridge	1	4	1	3	0	0	0	0
73	ridge	1	11	5	6	0	0	0	0
74	ridge	1	0	0	0	0	0	0	0
75	ridge	1	5	0	2	3	0	0	0
76	ridge	1	9	1	5	1	0	1	1
77	ridge	1	13	7	5	0	0	0	1
78	ridge	1	48	34	5	5	1	0	3
79	ridge	1	59	41	11	4	1	0	2
80	ridge	1	54	39	10	3	1	0	1

continue overleaf

81	ridge	1	54	22	15	12	2	2	1
82	ridge	1	28	7	17	2	1	1	0
83	ridge	1	45	37	5	3	0	0	0
84	ridge	1	47	26	14	4	3	0	0
85	ridge	1	24	10	9	5	0	0	0
86	ridge	1	29	24	5	0	0	0	0
87	ridge	1	36	27	6	1	2	0	0
88	ridge	1	21	14	4	2	0	1	0
89	ridge	1	49	26	9	12	0	1	1
90	ridge	1	54	30	3	5	2	2	12
91	ridge	1	32	9	5	5	6	5	2
92	ridge	1	3	1	0	0	0	0	2
93	ridge	1	12	4	0	2	1	2	3
94	ridge	1	48	27	10	5	1	2	3
95	ridge	1	27	23	3	0	1	0	0
96	ridge	1	30	22	3	1	2	1	1
97	ridge	1	44	30	7	5	2	0	0
98	ridge	1	19	17	2	0	0	0	0
99	ridge	1	38	11	8	7	2	5	5
100	ridge	1	5	5	0	0	0	0	0
Total			**1667**	**911**	**342**	**203**	**50**	**44**	**117**
Mean			**16.67**	**9.11**	**3.42**	**2.03**	**0.49**	**0.44**	**1.15**
Stdev			**23.166**	**15.941**	**5.339**	**3.264**	**1.04**	**1.104**	**2.118**

Appendix 11 : The number of plants in each height category distributed across three zones of mountain terrain at CH

Zones	No. of plants (density)	% density	Density											
			HC1		HC2		HC3		HC4		HC5		HC6	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
ridge	1273	76.36	661	39.65	267	16.02	167	10.02	41	2.46	43	2.58	94	5.64
valley	95	5.7	28	1.68	19	1.14	22	1.32	2	0.12	1	0.06	23	1.38
plateau	299	17.94	222	13.32	56	3.36	14	0.84	7	0.42	0	0	0	0
Total	**1667**	**100**	**911**	**54.65**	**342**	**20.52**	**203**	**12.18**	**50**	**3**	**44**	**2.64**	**117**	**7.02**

Appendix 12a : The number of epiphytic (E) and terrestrial (T) plants in the sample plots at CH and Mt. Purun

HC	CH		Mt. Purun	
	T	E	T	E
1	451	460	51	47
2	168	174	9	11
3	108	95	10	31
4	26	24	4	20
5	20	24	6	7
6	64	53	6	4
Total	**837**	**830**	**86**	**120**

Appendix 12b : The number of epiphytic (E) and terrestrial (T) plants distributed across three mountain zones in the sample plots at CH

Zones	CH	
	T	E
ridges	628	645
valley	22	73
plateau	187	112
Total	837	830

Appendix 13 : The chi-square test on the spatial distribution of male and female plants in the sample plots at Mt. Purun

No. of plants in plot	Observed frequency	Expected frequency	Chi-square
0	55	43.99	2.76
1	2	16.5	12.74
>1	7	3.52	3.44
Sum	64	64.01	18.94

$P<0.001$; $df = 1$

Appendix 14 : Preparation of solutions and procedures for anatomical studies

(a) CRAF III Fixative

30 ml of 1% chromic acid was mixed with 20ml of 10% acetic acid and stored in a bottle. 40% formaldehyde was mixed separately with 40 ml distilled water. Both solutions were stored in the refrigerator.

(b) Tertiary Butyl Alcohol Series

	H_2O	C_2H_5OH (95%)	TBA	C_2H_5OH (100%)
TBA1	50	40	10	-
TBA2	30	50	20	-
TBA3	15	50	35	-
TBA4	-	50	50	-
TBA5	-	-	75	25

Procedure:
Serial dehydration of the specimens were undertaken with 50% TBA (4 hours), 70% TBA (overnight), 85% (4 hours), 95% TBA (4 hours), 100% TBA (4 hours) and pure TBA (overnight).

(c) Safranin and Fast-Green
4 grams of the safranin dye was dissolved in 200 ml of methyl cellosolve. 100 ml of 95% alcohol and 100 ml of distilled water were added followed by 4 grams of sodium acetate in 8 ml formalin.

1 gram of Fast Green was added into a mixture containing 30 ml clove oil, 30 ml methyl cellosolve and 30% absolute alcohol and stirred thoroughly.

Staining Procedure :

Xylol	30 min
Xylol-Ethanol	15 mins
95 % Ethanol	10 mins
80% Ethanol	5 mins
70% Ethanol	5 mins
50% Ethanol	5 mins
1% Safranin	5 seconds
Water (3 rinses)	3 minutes
50 % Ethanol	2 minutes
70% Ethanol	2 minutes
95% Ethanol	2 minutes
0.5% Fast-Green	2 seconds
Xylol-Ethanol (1:1)	15 seconds
Xylol 1	15 minutes
Xylol 2	4 hours

Appendix 15 : ANOVA on the mean number of mature fruits produced by plants of different height categories

Height category (HC)			
3	4	6	5
22	38	0	22
30	36	28	
44	38		
15	24		
12			
21			
0			

ANOVA

Groups	Count	Sum	Average	Variance
HC3	7	144	20.57142857	194.619048
HC4	4	136	34	45.3333333
HC5	2	28	14	392
HC6	1	22	22	nil

Source Var.	SS	df	MS	F	P-value	F crit
Betw Groups	683.714286	3	227.9047619	1.34400449	0.3149117	3.708265695
Within Groups	1695.71429	10	169.5714286			
Total	2379.42857	13				

Appendix 16 : The flowering periodicity of the sample population at CH (November 1993-December 1994)

			Month (1993-1994)													
Plot	Tag no.	Sex	Nov.	Dec.	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
3	5	F														
6	42	M											+	+		
11	25	M														
13	5	M														
16	53	M							+	+						
16	77	F														
17	3	F											+	+	+	+
17	5	F							+	+	+	+	+	+	+	+
61	9	F									+	+	+	+	+	+
77	13	F								+	+	+	+	+	+	+
79	48	M								+	+					
79	53	M								+	+					
80	37	F														
81	13	F														
81	14	F														
81	19	F														
81	21	F														
91	14	F							+	+	+	+	+	+	+	+
94	47	M							+	+				+	+	+
96	10	M														
99	2	M							+	+						
99	3	M														
99	20	M														
99	33	M							+	+						

Appendix 17 : The mean number of weeks taken by the male and female inflorescences to reach anthesis and the mean number of weeks at anthesis

Replicate	No. of weeks to reach anthesis		No. of weeks at anthesis	
	Male	Female	Male	Female
R1	6	7	7	4
R2	7	7	4	5
R3	4	7	6	4
R4	5	8	6	4
R5	5	7	4	4
Mean	5.4	7.2	5.4	4.2
Stdev	1.140175	0.4472136	1.341641	0.447214

Appendix 18 : The time range of anther dehiscence in selected male inflorescences in the sample population at Mt. Purun

Replicate * (inflorescence)	Anther dehiscence time range (hours)
R1	0630 - 1330
R2	0645 - 1300
R3	0700 - 1700
R4	0700 - 1215
R5	0700 - 1400
R6	0700 - 1330
R7	0700 -1600
R8	0700 - 1630
R9	0700 - 1630
R10	1030 - 1700

* Each replicate consisted of an average of 28.5 flowers

Appendix 19 : The increment in the mean fruit length (cm.) of selected infructescences over a period of 11 weeks after anthesis

	Weeks after anthesis *										
	1	2	3	4	5	6	7	8	9	10	11
R1	0.5	0.6	0.7	0.9	1.4	1.7	2	2.2	2.3	2.3	2.3
R2	0.6	0.8	0.9	1.3	1.7	2	2	2	2	2	2
R3	0.5	0.8	1	1.3	1.7	2.2	2.2	2.3	2.3	2.3	2.3
Mean	0.53	0.73	0.87	1.17	1.6	1.97	2.07	2.17	2.2	2.2	2.2

Each replicate consisted of 12 fruits

* The dates varied between infructescences

Appendix 20 : The observed number of flowers at anthesis on selected dates in 1993 and 1994

Month	Date	No. of male flowers	No. of female flowers
December	30	7	n.a.
	31	5	n.a.
January	5	2	n.a.
	6	1	n.a.
	7	5	n.a.
	11	8	32
	12	2	89
	13	13	89
	14	8	89
	15	4	89
	16	1	89
	17	3	89
	18	6	89
	19	10	89
	20	11	89
	21	2	89
	22	2	89
	23	2	89
	24	1	89
	25	7	89
	26	2	89
	27	4	89
	28	4	89
	2	6	89
February	3	1	89
	4	3	89
	5	4	89
	6	5	89
	7	1	89
	8	3	89
	9	n.a.	32
	11	n.a.	n.a.
	14	1	n.a.
	15	14	n.a.
	16	20	n.a.
	17	6	n.a.
March	1	7	n.a.
	2	29	n.a.
	4	16	n.a.
	5	1	n.a.
	7	2	n.a.
	8	3	n.a.
	9	1	n.a.
Total		**233**	
Mean		**5.7**	
Stdev		**5.82**	

Appendix 21a : The floral production of a male inflorescence and the anther dehsicence date of the first flower

Plant no.	Inflor. no.	Total no. flowers	No.of mat. flowers	% of mat. flowers	Date of anthesis
01	02	117	117	100	17/1/94
02	01	97	97	100	3/1/94
03	01	98	98	100	14/2/94
04	01	198	193	97.5	29/12/93
	02	162	161	99.4	3/4/94
	03	124	124	100	20/6/94
	04	144	144	100	28/11/94
05	01	122	122	100	18/1/94
	02	144	143	99.3	27/2/94
	03	122	122	100	20/6/94
	04	121	121	100	14/6/94
	05	100	26	26	n.a.
06	01	135	135	100	10/1/94
	02	108	108	100	20/6/94
07	01	120	120	100	31/1/94
	02	108	108	100	22/5/94
	03	118	118	100	26/9/94
	04	95	0	0	9/1/95
08	01	102	102	100	13/2/94
09	01	80	80	100	14/2/94
	02	66	66	100	6/6/94
	03	68	37	54.4	3/1/95
010	01	135	135	100	14/3/94
011	01	192	177	92.2	2/5/94
	02	170	170	100	5/11/94
012	01	137	137	100	25/7/94
Total		**3183**	**2961**		
Mean		**122.42308**	**113.9**	**91.1**	
Stdev		**32.971713**	**44.69**	**24.95**	
Range		**66-198**	**0-193**	**0-100**	

Appendix 21b : The floral production of a female inflorescence and its 'putative stigma receptivity' date

Plant. no.	Inflor. no.	No. of flowers	Date of anthesis
01	02	48	10/1/94
	03	41	12/3/94
05	02	42	10/1/94
06	02	41	10/1/94
08	02	35	20/12/93
	03	29	20/5/94
	04	38	20/12/94
09	02	55	12/1/94
012	02	64	n.a.
	03	66	10/1/94
013	02	44	5/4/94
	03	42	4/8/94
	04	34	n.a.
014	01	67	10/1/94
	02	42	16/5/94
015	01	50	17/1/94
	02	70	12/1/94
017	01	82	11/1/94
	02	57	5/4/94
018	01	43	10/1/94
020	01	52	9/3/94
021	01	68	31/1/95
022	01	62	n.a.
	02	52	12/5/94
	03	36	31/10/94
023	01	35	27/1/95
025	01	62	27/12/94
026	02	38	5/12/94
027	02	28	5/12/94
028	02	54	21/2/95
029	01	48	9/1/95
030	01	21	n.a.
031	01	25	27/12/94
032	01	17	20/12/94
Total		**1588**	
Mean		**46.7**	
Stdev		**15.19874279**	
Range		**17-82**	

Appendix 21c : The fruit production in the sample population at Mt. Purun

Plant no.	Inflor. no.	Total no. of fruits	No. of mat. fruits	No. of immat. fruits	% Immature	Reprod. efficiency	No. weeks to maturity
01	01	51	43	8	15.6	84.4	n.a.
	02	48	38	10	20.8	79.2	25
	03 *	41	16	25	60.9	n.a.	27
02	01	44	44	0	0	100	n.a.
03	02	46	46	0	0	100	n.a.
04	02	34	28	6	21.4	78.6	n.a.
05	01	50	50	0	0	100	n.a.
	02	43	0	43	100	0	nil
06	01	38	36	2	5.2	94.8	n.a.
	02 **	41	0	41	100	n.a.	nil
07	01	33	33	0	0	100	n.a.
08	01	34	34	0	0	100	n.a.
	02	35	11	24	68.6	31.4	25
	03	29	0	29	100	0	nil
	04 **	28	0	28	100	n.a.	nil
09	01	48	44	4	8.3	91.7	n.a.
010	01	67	65	2	3	97	n.a.
	02	27	0	27	100	0	nil
012	01	64	59	5	7.8	92.2	n.a.
	02 *	66	3	63	95.5	n.a.	24
013	01	41	37	4	9.8	90.2	n.a.
	02 *	44	10	34	77.3	n.a	24
	03 *	42	8	34	80.9	n.a	25
	04	35	35	0	0	100	23
014	01	62	0	62	100	0	nil
	02 *	42	1	41	97.6	n.a.	23
015	01 *	50	0	50	100	n.a.	nil
	02	70	0	70	100	0	nil
017	01 **	62	0	62	100	n.a.	nil
	02 *	57	0	57	100	n.a.	nil
018	01 *	43	24	19	44.2	n.a.	30
020	01 *	52	10	42	81	n.a.	17
021	01	68	0	68	100	0	nil
022	01	62	34	28	45.2	54.8	n.a.
	02 *	52	2	50	96.1	n.a.	25
	03	36	0	36	100	0	nil
023	01	35	0	35	100	0	nil
025	01	56	38	18	32.1	67.9	20
026	02	38	38	0	0	100	22
027	02	26	0	26	100	0	nil
028	02 **	66	0	66	100	n.a.	nil
029	01	48	44	4	8.3	91.7	19
030	01	21	0	21	100	0	nil
031	01	24	21	5	20.8	87.5	20
032	01	17	12	5	29.4	70.6	21
Total		**176**	**864**				
Mean		**35.2**	**23.94**			**59.23**	**23.24**
Stdev		**21.04**	**20.784**			**39.991**	**3.243**

NB : * = hand pollination

** = control bagging

Appendix 21d : The seed set for selected fruits developed from open and hand pollination in the sample population at Mt. Purun

Rep.	Open pollination		Hand pollination *	
	No mature	No. immature	No mature	No. immature
1	98	48	0	129
2	84	57	14	128
3	93	42	125	10
4	110	24	40	99
5	86	45	116	21
6	95	41	46	87
7	91	47	19	122
8	104	29	148	7
9	81	52	130	14
10	89	45	143	6
11	92	48		
12	96	45		
13	84	49		
14	62	78		
15	127	10		
16	75	55		
17	78	61		
18	116	19		
19	94	51		
20	95	38		
Sum	**1850**	**884**	**781**	**623**
Mean	**92.5**	**44.2**	**78.1**	**62.3**
Stdev	**14.5439443**	**15.167833**	**59.264848**	**55.06370049**

* Ten replicates only

Appendix 22 : Regression analysis of the nearest male neighbour and the reproductive efficiency of female plants in the sample population at Mt. Purun

Regression Statistics	
Multiple R	0.1237395
R Square	0.01531146
Adjusted R Square	-0.0667459
Standard Error	31.6689647
Observations	14

ANOVA

	df	*SS*	*MS*	*F*	*Signific F*
Regression	1	187.1401	187.14	0.18659	0.673426
Residual	12	12035.08	1002.92		
Total	13	12222.22			

	Coefficients	*Std Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95%*	*Upper 95%*
Intercept	66.7332757	15.93725	4.18725	0.00126	32.009	101.4575533	32.00899815	101.4575533
X Variable 1	-0.3423413	0.792519	-0.432	0.67343	-2.06909	1.384409511	-2.06909211	1.384409511

Appendix 23 : The frequency and foraging activities of flower visitors in the sample population at Mt. Purun

30 December 1993
0950 hours : Carrion fly (Diptera) visited buds and flowers of male 0401. It moved systematically from one flower to another, seeking nectar at the inner and occasionally at the outer surface of the teals. It clambered up to the anther several times to feed on pollen grains, thus collecting the grains on its legs, abdomen and thorax. After feeding, it started to clean off the pollen grain from its legs. The fore legs were brought close to its mouth but whether the fly continued to feed on the pollen grains or was merely brushing off the grains remained unknown. The hind legs were rubbed together to remove the grains which were discarded in clumps. It did not feed on the clumps.

12 January 1994
1145 hours : A Muscidae fly (order Diptera, suborder Cyclorrhapha) foraged on buds, fresh and old flowers of male 0201. Visit lasted 5 minutes.
1300 hours : A Muscidae fly foraging on opened flowers of female 01502. It foraged quickly among flowers, moving in a circular motion, concentrating mainly on the inner tepals and ovary. Due to the proximity of the flowers, it often had to clamber over the stigma. The visit lasted 10 minutes.
1330 hours : The same species of fly foraged on the stigmatic surface of flowers of female 01502. It also foraged on buds and partially opened flowers. Visit lasted 3 minutes.
1337 hours : The same fly foraged on opened flowers of female 01502. Visit lasted 10 minutes.

13 January 1994
1038 hours : A Halictid bee (Hymenoptera) foraged on buds and flowers of male 0201. It browsed quickly among flowers for nectar at the ovary and the inner surface of tepals.
1316 hours : A Muscidae fly visited female 01502, probing for nectar on the inner surface of tepals. Visit lasted 3 mins.
1400 hours : A Halictid bee foraged on flowers of male 0601. Visit lasted 1 minute.

14 January 1994
1210 hours : A mosquito (Diptera, suborder, Nematocera) visited flowers of female 0502. It remained perched on a tepal for 40 minutes. There was no signs of feeding activity.
1650 hours : A spider visited old flowers of male 0601. The pollen grains were clearly visible on its thorax, abdomen and legs.

15 January 1994
1150 hours : The flower fly (Syrphidae) (Diptera, suborder Cyclorrhapha) visited flowers of male 0601. Walking from flower to flower in a horizontal circular plane, and with its legs far apart, it foraged for nectar on both surfaces of tepals. It often made repeated visit to the same flowers. It also foraged on old flowers. Not the entire visit was spent in feeding; half the time it remained perched on the inflorescence. The pollen grains were visible on its legs but they rapidly dropped off as it moves from one part of the flower to another.
1205 hours : The flower fly visited a dehisced anther of 0601. Even though it did not forage for food, it inadvertently gathered pollen grains on its legs. Pollen grains were transferred to other parts of its anatomy by the act of rubbing its hind legs against the abdomen. The visit lasted for several minutes. A return visit was made at 1216 hours, browsing amongst flowers for a period four minutes.

1317 hours : A Muscidae fly foraged on flowers of female 01502. It clambered over the ovaries in a horizontal circular plane, seeking nectar from the inner surface of tepals and ovaries. Even though it made repeated visits to the same flowers, it did not seem to have any flower preferences. It also foraged on partially opened flowers. After 13 minutes of observation, the visit was terminated with its capture.

20 January 1994
1045 hours : A Muscidae fly visited the bagged female 0902.
1110 hours : A Muscidae fly visited fruits of female 0102.

2-4 February 1994
No insect visitors throughout the day

8 February 1994
No insect visitors throughout the day

16 February 1994
0930 hours : A small crab found on male 0102. It could be the culprit that destroyed some flowers and buds of the inflorescence.
1300 hours : A plant hopper (Dictyopharidae) visited buds of male 0502.

2 March 1994
1345 hours : A Tiphiid wasp (Tiphiidae) wasp visited flowers of male 0502. During its forage, it collected pollen grains on its legs, abdomen and thorax. The visit lasted 10 minutes.

12 April 1994
2015 hours : A cockroach (Blattidae) visited flowers of male 01001. The forage lasted 20 minutes.
2230 hours : A cockroach visited flowers of 01001. The forage lasted 25 minutes.

20 April 1994
1355 hours : A Halictid bee visited flowers of male 0402. The forage lasted 15 mins.

22 April 1994
1645 hours : A flower fly (Syrphidae) visited flowers of male 0402. The forage lasted three minutes.

Ants (Formicidae) were the most frequent visitor on male and female flowers. Often, a group of them were observed on the inflorescence, busily moving from flower to flower foraging mainly on nectar of tepals and stigma. In the female inflorescence, they walked along the tepals to get to the next flower while in the male inflorescence, they moved from flower to flower using the pedicel and peduncle. They also used the pedicel and peduncle as the way out.

Appendix 24 : ANOVA on the mean length of fruits developed from treatments using different timings of pollen application

Timing (days after anthesis)				
14	21	28	35	no application
1.8	1.6	1.3	1.2	1.2
2.2	1.3	1.1	1.2	1.2
2.4	1.6	1.3	1.2	1.4
2.3	1.2	1.2	1.5	2.2
1.2	1.2	1.4	1.2	1.5
1.6	1.3	1.7	1.2	1.4
1.3	1.5	1.8	1.2	1.3
1.3	1.2	1.5	1.3	1.4
2	1.4	2.2	1.4	1.3
1.5	1.6	1.5	1.5	1.3
1.5	2.1	1.3	1.6	1.3
1.5	1.8	2.4	1.5	1.4
1.8	1.4	2	1.7	1.5
1.5	1.5		1.5	1
2.6	1.1		1.3	1
2.7	2.1		1	1.1
2.6	1.7		1.5	1.3
	1.7		1.3	
			1.7	

NB : Number of replicates differed between treatments
Fruit length in cm

ANOVA

Groups	Count	Sum	Average	Variance
Day 14	17	31.8	1.870588	0.254705882
Day 21	18	27.3	1.516667	0.085
Day 28	13	20.7	1.592308	0.162435897
Day 35	19	26	1.368421	0.037836257
no applic.	17	22.8	1.341176	0.071323529

Source Var	SS	df	MS	F	P-value	F crit
Betw Groups	3.12777	4	0.781942	6.648201623	0.000115	2.48736853
Within Groups	9.291754	79	0.117617			
Total	12.41952	83				

Appendix 25 : ANOVA on the mean initial mc for two seed batches derived either from one or from many plants

Batch 1*	Batch 2 **
12.25	9.97
11.8	9.93
10.09	9.99
8.34	9.18
9.62	9.82
12.63	9.3
10.51	8.02
8.25	8.45
7.17	10.07
11.77	9.77
8.46	11.45
8.54	10.5
8.98	8.48
8.59	11.38
8.93	12.31
8.98	10.06
13.89	10.53
9.59	9.77
12.5	10.05
10.66	10.43

* derived from one plant
** derived from many plants

ANOVA

Groups	Count	Sum	Average	Variance
Lot 1	20	201.55	10.0775	3.377609
Lot 2	20	199.46	9.973	1.052369

Source Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.1092025	1	0.109202	0.049302	0.825472	4.098169
Within Groups	84.169595	38	2.214989			
Total	84.2787975	39				

Appendix 26 : ANOVA on the transformed mean germination percentage for seed batches stored under a range of conditions

Effect	df Effect	MS Effect	df Error	MS Error	F	P level
Code	20	0.034414	63	0.006646	5.177774	0

Marked effects significant at P<0.05

Treatment code *	ASIN_%		
	Mean	Stdev	N
A	0.884175	0.07366	4
B	0.902825	0.087279	4
C	0.9974	0.04853	4
D	1.0487	0.057649	4
E	0.88425	0.072283	4
F	0.864425	0.096429	4
G	0.9309	0.069569	4
H	0.855925	0.052893	4
I	0.93685	0.0835	4
J	0.90685	0.038496	4
K	0.84315	0.045921	4
L	0.861725	0.100499	4
M	1.0081	0.094803	4
N	0.747475	0.09359	4
O	0.9787	0.060084	4
P	1.007925	0.029174	4
Q	0.9359	0.059747	4
R	1.01185	0.063638	4
S	1.1897	0.056484	4
T	0.96055	0.199647	4
U	0.871475	0.071788	4
AllGroups	nil	0.11548884	

* see Table 6 for treatment code

Appendix 27 : ANOVA on the mean germination time for seed batches stored under a range of conditions

Effect	df Effect	MS Effect	df Effect	MS Error	Error	F	plevel
Code	20	128	0.1378	55	14.11	9.08157	0

Marked effects significant at P<0.05

Treatment code *	TIME		
	Mean	Stdev	N
A	22.20667	4.249486	3
B	34.5725	1.422565	4
C	22.15	3.8371	4
D	28.7175	1.023633	4
E	20.425	6.38664	4
F	32.71	4.031414	3
G	20.225	8.899579	4
H	35.6325	1.380613	4
I	22.4	4.804859	4
J	28.89	3.134943	4
K	23.13333	3.308071	3
L	31.61333	2.967917	3
M	23.675	2.873296	4
N	36.92	0	1
O	21.3	3.4	4
P	29.325	0.695965	4
Q	17.5	0.8	4
R	29.7075	0.537858	4
S	17.3275	1.761011	4
T	18.30333	0.936287	3
U	30.505	5.011331	4
All Groups	nil	6.67212	76

* see Table 6 for treatment code

Appendix 28 : ANOVA on the mean cumulative germination percentages* of seed batches cultured onto various basal media

ANOVA

Groups	Count	Sum	Average	Variance
MS	4	153.21	38.3025	61.526025
GB5	4	173.98	43.495	59.903567
NN	4	180.09	45.0225	39.246892
SH	4	159.48	39.87	42.141067
VW	4	174.23	43.5575	13.814492
AS	4	237.73	59.4325	33.437625

Source Variation	SS	df	MS	F	P-value	F crit
Between Groups	1135.2	5	227.0398	5.4474369	0.003176	2.77285
Within Groups	750.21	18	41.67828			
Total	1885.4	23				

* values are in arc-sine transformed percentages

Appendix 29 : The mean germination time taken for a seed batch* to achieve at least 50% germination

Medium	MS	GB5	NN	SH	VW	AS	in vivo **
R1	n.a.	28.5	34	n.a.	34.5	36.6	18.29
R2	n.a.	34.2	n.a.	n.a.	n.a.	30.5	15.3
R3	35	n.a.	32	n.a.	40.15	28.6	19.22
R4	36.5	n.a.	31.2	33.5	n.a.	25	16.5
mean	n.a.	n.a.	n.a.	n.a.	n.a.	30.175	17.3275
s.d.	n.a.	n.a.	n.a.	n.a.	n.a.	4.852748	1.7610106

* n = 20

**results obtained from the germination of fresh seeds from different fruits of the same plant

n.a. = replicates did not achieve 50% germination by the end of the observation period

Appendix 30a : ANOVA on the survival percentages* of seedlings cultured onto various basal media and BAP concentations

ANOVA

Groups	Concentration(M)	Count	Sum	Average	Variance
MS-full strength	0	2	0	0	0
	10x-6	2	0	0	0
	5x10-6	2	0	0	0
	10x-5	2	0	0	0
MS-half strength	0	2	90	45	66.5858
	10x-6	2	102.66	51.33	292.82
	5x10-6	2	102.66	51.33	292.82
	10x-5	2	78.46	39.23	0
GB5-Full strength	0	2	65.8	32.9	80.1378
	10x-6	2	26.57	13.285	352.9825
	5x10-6	2	78.46	39.23	0
	10x-5	2	39.23	19.615	769.4965
GB5-Half strength	0	2	102.66	51.33	292.82
	10x-6	2	78.46	39.23	0
	5x10-6	2	102.66	51.33	292.82
	10x-5	2	39.23	19.615	769.4965
NN	0	2	78.46	39.23	0
	10x-6	2	65.8	32.9	80.1378
	5x10-6	2	26.57	13.285	352.9825
	10x-5	2	53.14	26.57	0
SH	0	2	78.46	39.23	0
	10x-6	2	102.66	51.33	292.82
	5x10-6	2	53.14	26.57	0
	10x-5	2	26.57	13.285	352.9825
VW	0	2	26.57	13.285	352.9825
	10x-6	2	39.23	19.615	769.4965
	5x10-6	2	53.14	26.57	0
	10x-5	2	26.57	13.285	352.9825
AS	0	2	63.43	31.715	2011.682
	10x-6	2	39.23	19.615	769.4965
	5x10-6	2	153.43	76.715	352.9825
	10x-5	2	116.57	58.285	2011.682

Source Variation	SS	df	MS	F	P-value	F crit
Between Groups	22781.47354	31	734.886	2.155446	0.017058	1.81038
Within Groups	10910.2068	32	340.944			
Total	33691.68034	63				

* values are arc-sine transformed percentages

Appendix 30b : ANOVA on the mean number of shoot buds* proliferating from cotyledonary seedlings cultured onto various basal media and BAP concentrations

ANOVA

Groups	Concentration (M)	Count	Sum	Average	Variance
MS-full strength	0	2	1.41421	0.707107	0
	10x-6	2	1.41421	0.707107	0
	5x10-6	2	1.41421	0.707107	0
	10x-5	2	1.41421	0.707107	0
MS-half strength	0	2	2.44949	1.224745	0
	10x-6	2	2.44949	1.224745	0
	5x10-6	2	3.05423	1.527115	0.005837
	10x-5	2	3.70246	1.85123	0.145898
GB5-Full strength	0	2	2.44949	1.224745	0
	10x-6	2	1.93185	0.965926	0.133975
	5x10-6	2	2.44949	1.224745	0
	10x-5	2	1.93185	0.965926	0.133975
GB5-Half strength	0	2	2.44949	1.224745	0
	10x-6	2	2.80588	1.402942	0.063508
	5x10-6	2	2.44949	1.224745	0
	10x-5	2	3.05231	1.526157	1.341688
NN	0	2	2.44949	1.224745	0
	10x-6	2	2.44949	1.224745	0
	5x10-6	2	1.93185	0.965926	0.133975
	10x-5	2	2.80588	1.402942	0.063508
SH	0	2	2.44949	1.224745	0
	10x-6	2	2.44949	1.224745	0
	5x10-6	2	2.44949	1.224745	0
	10x-5	2	1.93185	0.965926	0.133975
VW	0	2	1.93185	0.965926	0.133975
	10x-6	2	1.93185	0.965926	0.133975
	5x10-6	2	2.44949	1.224745	0
	10x-5	2	1.93185	0.965926	0.133975
AS	0	2	1.93185	0.965926	0.133975
	10x-6	2	2.28825	1.144123	0.381966
	5x10-6	2	2.80588	1.402942	0.063508
	10x-5	2	3.45197	1.725984	0.04196

Source Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.831886637	31	0.15587	1.568639	0.105391	1.81038
Within Groups	3.179670569	32	0.09936			
Total	8.011557206	63				

* values are in square-root transformed numbers

Appendix 31 : ANOVA on the mean number of shoot buds* proliferating from apical shoots cultured onto various basal media and BAP concentrations

ANOVA

Groups	Concentration(M)	Count	Sum	Average	Variance
MS-full strength	0	2	1.4142136	0.7071068	0
	10x-6	2	2.2882456	1.1441228	0.381966
	5x10-6	2	1.9318517	0.9659258	0.1339746
	10x-5	2	2.2882456	1.1441228	0.381966
MS-half strength	0	2	1.4142136	0.7071068	0
	10x-6	2	2.8058837	1.4029419	0.0635083
	5x10-6	2	3.1622777	1.5811388	0
	10x-5	2	3.4519675	1.7259838	0.0419601
GB5-Full strength	0	2	1.4142136	0.7071068	0
	10x-6	2	1.4142136	0.7071068	0
	5x10-6	2	2.2882456	1.1441228	0.381966
	10x-5	2	2.1213203	1.0606602	0.25
GB5-Half strength	0	2	1.4142136	0.7071068	0
	10x-6	2	3.3131896	1.6565948	0.0113872
	5x10-6	2	1.4142136	0.7071068	0
	10x-5	2	1.4142136	0.7071068	0
NN	0	2	1.4142136	0.7071068	0
	10x-6	2	1.4142136	0.7071068	0
	5x10-6	2	1.4142136	0.7071068	0
	10x-5	2	1.4142136	0.7071068	0
SH	0	2	1.4142136	0.7071068	0
	10x-6	2	1.4142136	0.7071068	0
	5x10-6	2	2.1213203	1.0606602	0.25
	10x-5	2	1.4142136	0.7071068	0
VW	0	2	1.4142136	0.7071068	0
	10x-6	2	1.4142136	0.7071068	0
	5x10-6	2	1.9318517	0.9659258	0.1339746
	10x-5	2	1.4142136	0.7071068	0
AS	0	2	1.4142136	0.7071068	0
	10x-6	2	2.6389584	1.3194792	0.0179492
	5x10-6	2	1.9318517	0.9659258	0.1339746
	10x-5	2	1.4142136	0.7071068	0

Source Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.158582357	31	0.1986639	2.9126586	0.001753	1.810378
Within Groups	2.182626663	32	0.0682071			
Total	8.34120902	63				

* values are in square-root transformed numbers

Appendix 32 : ANOVA on the mean number of shoot buds* proliferating from cotyledonary seedlings cultured onto media supplemented with various cytokinins and its concentrations

ANOVA

Groups	Concentration (M)	Count	Sum	Average	Variance
2-iP	10x-6	4	3.346065	0.8365163	0.0669873
	5x10-6	4	2.828427	0.7071068	0
	10x-5	3	2.638958	0.8796528	0.0893164
	2.5x10-5	4	4.242641	1.0606602	0.5
	5x10-5	4	3.346065	0.8365163	0.0669873
Kinetin	10x-6	4	2.828427	0.7071068	0
	5x10-6	4	4.242641	1.0606602	0.5
	10x-5	4	4.242641	1.0606602	0.5
	2.5x10-5	4	3.346065	0.8365163	0.0669873
	5x10-5	4	4.509787	1.1274468	0.3051517
BAP	10x-6	4	5.255373	1.3138434	0.0317542
	5x10-6	4	7.292133	1.8230332	0.0420664
	10x-5	4	7.969459	1.9923647	0.1139774
	2.5x10-5	4	7.146378	1.7865945	0.5774399
	5x10-5	4	10.68258	2.6706445	0.0568769

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	18.06960835	14	1.290686	6.5552479	7.012E-07	1.923574
Within Groups	8.663318078	44	0.196894			
Total	26.73292643	58				

* values are in square-root transformed numbers

Appendix 33 : Survival percentages of cotyledonary seedlings cultured onto media supplemented with various auxin concentrations and incubated in light/dark

Medium	Auxin	Concentration (M)	Light/dark	% survival*
MS-half	2,4-D	0	+	100
			-	0
		10x-6	+	20
			-	0
		5x10-6	+	0
			-	0
		10x-5	+	0
			-	0
		2.5x10-5	+	0
			-	0
		5x10-5	+	0
			-	0
	2,4,5-T	0	+	n.a.
			-	n.a.
		10x-6	+	40
			-	0
		5x10-6	+	0
			-	0
		10x-5	+	0
			-	0
		2.5x10-5	+	0
			-	0
		5x10-5	+	0
			-	0

* After 2 months, no callus was observed on the seedlings that survived.
+ Incubated under 12 hours photoperiod with PAR 12-15
- Incubated in darkness throughout the duration of observation

Number of seedlings per replicate = 10
Number of replicates = 5 petri dishes
Incubated at 23-26 C, RH 67-75%